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CEPHALIN.

VII. THE GLYCEROPHOSPHORIC ACID OF CEPHALIN.

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(From the Laboratories of The Rockefeller Institute for Medical Research)

The generally accepted structural formula of cephalin is as follows:

$$H_2C - O - COC_{17}H_{31}$$
$$|$$
$$H C - O - COC_{17}H_{15}$$
$$|$$
$$H_2C - O - P = O$$
$$OH \quad O - CH_2CH_2NH_2$$
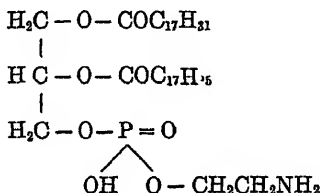
This conception presumes the existence of glycerophosphoric acid in the molecule of cephalin. Thudichum,[1] who discovered cephalin, also was the first to make this assumption. Every other worker who followed Thudichum made similar assumptions. Parnas[2] was the first to call attention to the lack of evidence in favor of the theory, though admitting the possibility of its correctness. MacLean in his monograph on "Lecithin and allied substances" presents a very lucid summary of the weak points of the theory.

The main objection to the conclusions of the older writers lies in the fact that the material employed by them was not a pure cephalin but a mixture of this substance with lecithin.

In recent years, Frankel and Dimitz[3] claimed to have identified glycerophosphoric acid as a component of cephalin. Unfortunately the evidence presented by these investigators is not con-

[1] Thudichum, J L W , The chemical constitution of the brain, London, 1884
[2] Parnas, J , *Biochem Z* , 1909, xxii, 411
[3] Frankel, S , and Dimitz, L , *Biochem Z* , 1909, xxi, 337.

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vincing since they have failed to substantiate their claim with satisfactory analytical data. The analytical data are limited to one element; namely, barium.

Regardless of the meager proof of the nature of their substance, Frankel and Dimitz have laid claim to a second very important discovery; namely, that the glycerophosphoric acid derived from cephalin was the optical isomer of the corresponding substance occurring in lecithin

Willstätter and Ludecke[4] found that the glycerophosphoric acid obtained from lecithin was optically active and levorotary. The substance obtained by Frankel and Dimitz was dextrorotary, and hence followed the claim that it was isomeric to the substance described by Willstätter and Lüdecke.

The presence of glycerophosphoric acid in the cephalin molecule seemed *a priori* quite probable in the light of facts discovered in recent years. Simultaneously, Foster[5] and Levene and West[6] have shown that glycerol is present in cephalin in the quantity required by the generally accepted theory.

Furthermore, it was found that the basic part of cephalin is amino ethyl alcohol, and that in this base the alcoholic group only is substituted, the amino group remaining free.

Theoretically there are only two alternative ways in which glycerol may be attached to the phosphoric acid, either directly (forming glycerophosphoric acid) or indirectly. If the linking were indirect, then the glycerol would be attached to the aminoethanol. If that were so, then the amino group could not be free in the molecule of cephalin. Thus the presence of glycerophosphoric acid in cephalin could have been accepted even before the substance was actually isolated, although for the final proof, the isolation of the substance would have been desirable. On the other hand, without isolating the substance there was no way to reach a decision as to which of the possible isomers of glycerophosphoric acid is the one present in the molecule of cephalin. True, the optical rotation of hydrolecithin and of hydrocephalin is practically identical in direction and magnitude. Levene and West[7] have pointed out that this fact may be

[4] Willstätter, R., and Lüdecke, K., *Ber chem Ges*, 1904, xxxvii, 3753

[5] Foster, M L, *J. Biol Chem*, 1915, xx, 403

[6] Levene, P. A, and West, C J, *J Biol Chem*, 1916, xxiv, 50

[7] Levene, P A, and West, C J, *J Biol Chem*, 1918, xxxv, 287

interpreted to signify that the glycerophosphoric acids in the two substances are identical. This conclusion contradicts the theory of Frankel and Dimitz. Hence it became necessary to choose between the two views, and, in order to make the choice intelligently, an effort was made to isolate the glycerophosphoric acid of the cephalin molecule.

Glycerophosphoric Acid from Lecithin.

The present communication contains a report on the preparation of glycerophosphoric acid from both cephalin and lecithin. The latter was prepared for the following reasons First, Willstätter and Ludecke[4] stated that the magnitude of the optical rotation of the glycerophosphoric acid from lecithin differed depending on the mode of preparation. Second, the directions for the preparation of the substances given by these authors are so meager that any one repeating the work could have no assurance that he was following exactly the conditions of Willstatter and Ludecke. Under such circumstances if the glycerophosphoric acid prepared by us from cephalin was found different from the one of Willstatter and Ludecke, the difference could have been ascribed not to the inherent properties of the substance, but to the manner of its preparation.

The glycerophosphoric acid obtained by us from lecithin resembled the one obtained by Willstatter and Ludecke, in respect to the direction of its rotation. It was levorotary. However, the magnitude of the rotation of our substance was lower than the maximum rotation found by the earlier workers. The differences are probably due to differences in handling, since all the samples were partially racemized.

The maximum rotation found by us was $[\alpha]_D^{20} = -0.74$, by the other writers $[\alpha]_D^{20} = -1.71$

In a general way the crude substance was prepared in the manner indicated by Willstätter and Ludecke. However, for purification it was found convenient to convert the barium salt into the lead salt, to purify the lead salt, and then to reconvert this into the barium salt.

As a rule, the hydrolysis was brought about at room temperature; however, for the sake of comparison one experiment was

performed by boiling the hydrolysis mixture.   This variation in
the condition of hydrolysis remained without influence on the
rotatory power of the resulting substance.

Hydrolysis of Cephalin.

After the most favorable conditions of hydrolysis of lecithin
had been established, they were applied to the hydrolysis
of cephalin   The result was practically identical.   The glycero-
phosphoric acid obtained from cephalin, purified through con-
version of the crude barium salt into the lead salt with subsequent
reconversion of the latter into the barium salt, possessed the
optical rotation of the same magnitude as the acid from lecithin,
namely, $[\alpha]_D^{20} = -0.69$.

In this respect the result obtained by us is new.   It establishes
the fact that glycerophosphoric acid enters into the structure
of cephalin, and further that the acid is identical with that
present in lecithin   However, it is worthy of note that while
the crude barium glycerophosphate obtained from lecithin always
showed a levorotation, though of a comparatively low magnitude,
the barium glycerophosphate from cephalin showed dextrorotation.
The magnitude of this rotation was progressively descending on
purification of the substance.   All the dextrorotary samples were
found to contain nitrogen, and the purification of the substance
which led to the fall of the dextrorotation also led to the diminu-
tion in the nitrogen content.   It is probable, therefore, that the
claim of Frankel and Dimitz to the discovery of a dextrorotary
glycerophosphoric acid from cephalin was an error brought
about by the fact that the substance analyzed by them was a
mixture of glycerophosphoric acid with some product of inter-
mediary hydrolysis

EXPERIMENTAL.

Barium Glycerophosphoric Acid from Lecithin.

Hydrolysis at Low Temperature.

75 gm of lecithin were obtained by the decomposition with
⁓um carbonate of lecithin.cadmium chloride containing
⁓mino nitrogen.   This material was ground under
⁓uniform emulsion was obtained, and then shaken

for 6 hours at room temperature with 1 liter of a saturated aqueous solution of barium hydroxide (the equivalent of $2\frac{1}{4}$ mols of $Ba(OH)_2$). After standing for 3 hours, the mixture was filtered and the excess of barium contained in the filtrate removed quantitatively with sulfuric acid. The resulting filtrate was concentrated to a small bulk under diminished pressure, and the barium salt precipitated by pouring the thin syrup into several volumes of absolute alcohol. After one purification by dissolving in a small quantity of water, filtering through bone-black, and reprecipitating with alcohol, the yield of this material was 7.5 gm.

The barium salt obtained in this way was subjected to two more purifications by solution in water and precipitation by alcohol. Finally it was twice dissolved in a minimum amount of water and allowed to stand until it separated as a mass of heavy granules and non-crystalline scales. These were filtered with suction, washed with alcohol, and dried under diminished pressure. Obtained in this manner, the salt, when dry, is a light tan, granular material, which dissolves in water giving a clear yellow solution. Though very soluble in cold water it is not hygroscopic, on warming the cold solution a fine amorphous powder is precipitated which does not dissolve completely as the solution is cooled. It is insoluble in alcohol, ether, and acetone. All rotations were made in 20 per cent aqueous solution A sample (No. 23) had the following composition. It contained no nitrogen.

0.1018 gm. of substance dried under diminished pressure at the temperature of zylene vapor gave on combustion 0 0472 gm. of CO_2, 0.0248 gm. of H_2O, and 0 0712 gm. of ash.

0 2000 gm of substance containing 7 36 per cent moisture gave 0 0666 mg of $Mg_2P_2O_7$

0 1000 gm of substance containing 7 36 per cent moisture gave 0 0668 mg. of $BaSO_4$

	Calculated for $C_2H_7O_4PBa$ per cent	Found No 23 per cent
C	11 71	12 64
H	2 29	2 72
P.	10 10	10 02
Ba	44 68	42 43

The rotation of the substance was as follows.

$$[\alpha]_D^{20} = \frac{-0\,075 \times 100}{1 \times 18.42} = -0.41.$$

As our investigation progressed, we found that the method of reprecipitation described above was not effective for the complete purification of the salts, as shown by their continued retention of nitrogen, and the process described below was applied to all our later products.

2 gm. of substance (No. 23) were dissolved in water. A 25 per cent aqueous solution of neutral lead acetate was added until precipitation was complete; the precipitate was filtered and thoroughly washed with water, alcohol, and ether. The lead salt thus isolated was suspended in water, and hydrogen sulfide passed through until the decomposition was complete. The lead sulfide was removed by filtration and the filtrate concentrated under diminished pressure to a small volume and made slightly alkaline to litmus with barium hydroxide. From the concentrated solution the barium salt was precipitated by adding several volumes of 95 per cent alcohol. The yield of this preparation, No. 63, was 1.5 gm.

When rapidly precipitated by alcohol, the barium salt separates in white amorphous flakes, which dry to a gleaming white powder. The color reported in the foregoing experiment is apparently due to an impurity as all samples obtained analytically pure were white when dry, and very readily soluble in water, giving colorless, water-clear solutions. If the salt after filtration is not dried either by washing with alcohol and ether or by rapid desiccation, the amorphous flakes undergo an apparent fusion and dry to a mass of colorless granules and scales This granular form can also be obtained by precipitating the salt from a very concentrated aqueous solution by a gradual addition of alcohol.

0 101 gm of substance dried under diminished pressure at temperature of xylene vapor gave on combustion 0 0438 gm of CO_2, 0 0194 gm of H_2O, and 0 0742 gm of ash

0.2786 gm of substance containing 9 11 per cent water gave 0 0912 gm of $Mg_2P_2O_7$.

	Calculated for $C_3H_7O_6PBa$ per cent	Found No 63 per cent
C .	11 71	11.84
H . .	2 29	2 15
P	10 10	10 00
Ba	44 68	44 03

The rotation of No. 63 is

$$[\alpha]_D^{20} = \frac{-0\ 11 \times 100}{1 \times 18\ 82} = -0\ 58.$$

Both granular and flocculent forms after air-drying contain water of crystallization, the value of which is not constant, though in general it approaches $1\frac{3}{4}$ mols of H_2O. The nature of the substance, however, does not permit one to attribute too much importance to this value. After preliminary drying under diminished pressure over sulfuric acid at room temperature, the loss of water on complete drying was more constant, corresponding to $C_3H_7O_6PBa\ H_2O$.

After air-drying, on desiccation over sulfuric acid under diminished pressure at the temperature of xylene vapor, 0 1108 gm. of No. 63 lost 0.0098 gm. of H_2O.

	Calculated for $C_3H_7O_6PBa\ 1\frac{3}{4}H_2O$. per cent	Found No 63 per cent
H_2O	9 30	8.84

The substance was dried for 24 to 48 hours under diminished pressure over sulfuric acid at room temperature. On further drying to constant weight at the temperature of xylene vapor, 0.1100 gm. of No. 56 lost 0.0060 gm. of H_2O and 0.1092 gm. of No. 57 lost 0 0058 gm. of H_2O.

	Calculated for $C_3H_7O_6PBa\ H_2O$ per cent	Found No. 56 per cent	No 57. per cent
H_2O	5 53	5 45	5 31

In order to be certain of the source of the glycerophosphoric acid, lecithin cadmium chloride was isolated from egg oil by the method recommended by Levene and West[8] for the preparation of pure lecithin. The amino content of this material was negligible. From its cadmium salt the lecithin was isolated by treatment with $(NH_4)_2CO_3$ in boiling 85 per cent alcoholic solution.

[8] Levene, P. A , and West, C. J , *J Biol. Chem*, 1918, xxxiv, 175.

75 gm. of this product were hydrolyzed with 1 liter of barium hydroxide solution, and the barium salt was isolated in the same manner as No. 23. 8 gm. of crude material were obtained from which the material most insoluble in alcohol was separated by repeated solution in water and fractional precipitation with small volumes of alcohol. After eighteen such reprecipitations, a substance (No. 55) of the following composition and analysis was obtained. It contained no nitrogen

0 200 gm of substance containing 5 03 per cent moisture gave 0 0706 gm of $Mg_2P_2O_7$

0 100 gm of substance containing 5 03 per cent moisture gave 0 0690 gm. of $BaSO_4$.

				Calculated for $C_3H_7O_4PBa$ per cent	Found No 55 per cent
N ...	.	.	. . . . .	0 00	0 00
P	.	.	..	10 10	10 36
Ba		.	. .	44 68	42 75

$$[\alpha]_D^{20} = \frac{- 0 \ 12 \times 100}{1 \times 18 \ 89} = -0 \ 63.$$

That a longer period of hydrolysis would give a better yield and not affect the rotation adversely seemed probable, and was proved by hydrolyzing 100 gm of lecithin for 16 hours with 1,350 cc. of a saturated barium hydroxide solution. When isolated by the method described for No 23 the yield of the crude material was 35 gm. (90 per cent of the theory). After three precipitations from alcohol this material (No. 56) had the following composition.

0.200 gm of substance containing 5 45 per cent moisture gave 0 0572 gm of $Mg_2P_2O_7$

0 100 gm of substance containing 5 45 per cent moisture gave 0 0664 gm of $BaSO_4$.

0 200 gm of substance containing 5 45 per cent moisture used for Kjeldahl nitrogen determination required 0 26 cc. of 0 1 N HCl.

				Calculated for $C_3H_7O_4PBa$ per cent	Found. No 56. per cent
N.	.	.	.. . .	0 00	0 18
P	.	. . .	..	10 10	8 43
Ba	. . . .	.	.	44 68	41 33

No. 56 was then further purified by precipitating the lead salt and decomposing this by the directions given above. The

resulting substance (No. 57) had the following composition and rotation. It contained no nitrogen.

0 200 gm. of substance containing 5 31 per cent moisture gave 0 0704 gm of $Mg_2P_2O_7$

0.100 gm of substance containing 5 31 per cent moisture gave 0 0680 gm of $BaSO_4$

			Calculated for $C_2H_7O_6PBa$ per cent	Found No 57 per cent
N	.	.	0 00	0 00
P .	.	..	10 10	10 38
Ba		. .	44 68	42 26

$$[\alpha]_D^{20} = \frac{-0\ 09 \times 100}{1 \times 19\ 36} = -0\ 46.$$

A second purification through the lead salt resulted in No. 60, whose rotation was

$$[\alpha]_D^{20} = \frac{-0\ 14 \times 100}{1 \times 18\ 92} = -0\ 74$$

A third purification by this method gave no further change in the rotation.

The final analysis after an additional precipitation with alcohol follows.

0.1026 gm. of substance dried *in vacuo* at temperature of xylene vapor gave on combustion 0 0482 gm of CO_2, 0 0248 gm of H_2O, and 0.0774 gm of ash.

0.200 gm of substance containing 7.06 per cent moisture gave 0 0696 gm. of $Mg_2P_2O_7$

				Calculated for $C_2H_7O_6PBa$ per cent	Found No 78. per cent
C	.. . .		. .	11 71	12 81
H	. .			2 29	2 70
P	.	. . .		10 10	10 43
Ba .	.		. .	44 68	44 87

Hydrolysis at Higher Temperature.

97 gm. of lecithin cadmium chloride were dissolved in 250 cc. of boiling 50 per cent alcohol and this solution was added to one containing 103 gm. (3¼ mols) of barium hydroxide in 1 liter of hot water. After 1 hour of gentle boiling the mixture was cooled and filtered. The excess of barium hydroxide was removed

from the filtrate by quantitative precipitation with sulfuric acid, and the filtered solution concentrated in vacuum to small bulk. The addition of an equal volume of 95 per cent alcohol precipitated the salt of glycerophosphoric acid, but the barium chloride formed in the course of the hydrolysis remained in solution. Thorough washing with 50 per cent alcohol removed all traces of chlorides from the filtered barium glycerophosphoric acid. After two preliminary precipitations by alcohol, the salt was converted into the lead salt, decomposed, and again reprecipitated as the barium salt by the directions given above.

The specific rotation of this substance was

$$[\alpha]_D^{20} = \frac{-0\ 03 \times 100}{1 \times 18\ 89} = -0\ 16$$

A second purification by conversion into the lead salt raised this rotation as follows

$$[\alpha]_D^{20} = \frac{-0\ 12 \times 100}{1 \times 18\ 89} = -0\ 63$$

On analysis 0 102 gm (No 69), dried under diminished pressure over sulfuric acid at temperature of xylene vapor, gave on combustion 0.0234 gm of H_2O, 0 0449 gm of CO_2, and 0 0724 gm of ash

0 300 gm. (No 69), containing 7 77 per cent moisture, gave 0 1021 gm of $Mg_2P_2O_7$

	Calculated for $C_3H_7O_6PBa$ per cent	Found. No 69 per cent
C	11 71	12 00
H	2 29	2 56
P	10 10	10 28
Ba	44 68	41 19

Barium Glycerophosphoric Acid from Cephalin.

Preparation of Cephalin.

Three samples of cephalin were used as the source of the glycerophosphoric salts described below. All were obtained from ox brains by six extractions with ether containing 5 per cent water. This material, after evaporation of the ether, was precipitated with acetone and the fats and saturated phosphatides were removed from the precipitate by repeatedly dissolving in ether,

removing all material insoluble in ether at $0°$, and reprecipitating by pouring into acetone. From the mixture of lecithin and cephalin thus obtained, the cephalin was separated by precipitating the ethereal solution with alcohol. The first sample of cephalin was purified by repeated precipitation by alcohol from ethereal solution and finally three precipitations from a solution in gasoline (B. P. 50-60°). The analysis of this material (No. 44) is given below

The second sample, after three precipitations from an ethereal solution by alcohol, was shaken with water until a uniform emulsion was obtained. From this the cephalin was precipitated by dilute hydrochloric acid, separated by centrifuging, and thoroughly washed with acetone   Further purification was effected by repeatedly dissolving either in ether (saturated with water at room temperature) or in gasoline (B P. 50-60°) and precipitating with alcohol. This material (No. 58) had the composition indicated below.

The third sample was prepared in the following manner. The crude cephalin was exhaustively extracted with alcohol at room temperature and then repeatedly reprecipitated by alcohol from ether or gasoline solution, until its amino content was that indicated in the analysis (No. 135) recorded below. Following this the material was emulsified with water, precipitated by hydrochloric acid, and the precipitate washed with alcohol.

No. 44

0.5 gm was dissolved in 5 cc. of glacial acetic acid

2 cc. of this solution for Kjeldahl determination required 3 40 cc of 0 1 N HCl = 0 00468 gm of N.

2 cc of this solution for Van Slyke determination gave 8 55 cc of N at $T° = 26°C$ and $P = 757 1$ mm ; N = 0 00476 gm.

$$\text{Ratio } \frac{NH_2N}{Total\,N} = \frac{1}{1}$$

No 58.

2 gm of cephalin were hydrolyzed with HCl, neutralized, and concentrated to 25 cc

5 cc. of this solution required for Kjeldahl determination 0 90 cc of 0.1 N HCl = 0 00126 gm. of N

2 cc. of this solution by Van Slyke determination gave 0.91 cc of N at $T° = 24°$ and $P = 755.9$ mm , N = 0 000505 gm.

$$\text{Ratio } \frac{NH_2N}{\text{Total N}} = \frac{1}{1}$$

No 135.

0 2 gm was dissolved in 10 cc. of glacial acetic acid

5 cc of this solution for Kjeldahl determination required 1 27 cc of 0 1 N HCl = 0 001778 gm. of N

1 cc of this solution for Van Slyke determination gave 0 65 cc of N at $T^\circ = 30^\circ$ and $P = 759\ 2$ mm ; N = 0 003505 gm

$$\text{Ratio } \frac{NH_2N}{\text{Total N}} = \frac{1}{1}$$

Hydrolysis of Cephalin.

75 gm. of cephalin (No. 44) were hydrolyzed by shaking for 9 hours at room temperature with a liter of saturated barium hydroxide solution.   The directions given for No. 23 were followed in working up the resulting mixture   On pouring the concentrated aqueous solution into alcohol a colloidal solution formed which was precipitated by the addition of a few cc of an aqueous solution of barium acetate.   After thirteen reprecipitations by alcohol from its aqueous solution, this material (No. 51) had the following composition and rotation.

0.200 gm of substance containing 5 67 per cent moisture gave 0 0556 gm of $Mg_2P_2O_7$.

0 100 gm of substance containing 5 67 per cent moisture gave 0 0662 gm of $BaSO_4$

0 200 gm of substance used for Kjeldahl determination required 1 12 cc. of 0 1 N HCl

	Calculated for $C_3H_7O_6PBa$	Found No 51
	per cent	per cent
N...	0 00	0 83
P	10 10	8 21
Ba	44 68	41 28

$$[\alpha]_D^{20} = \frac{+0\ 15 \times 100}{1 \times 18.88} = +0\ 80$$

No. 51 five times reprecipitated by alcohol still retained nitrogen.

0 200 gm. of substance (No 53) containing 9.28 per cent moisture required for Kjeldahl determination 0.77 cc of 0 1 N HCl

	Calculated for $C_3H_7O_4PBa$ per cent	Found No 53 per cent
N	0 00	0 59

Its rotation was

$$[\alpha]_D^{20} = \frac{+0\ 08 \times 100}{1 \times 18\ 20} = +0\ 44.$$

Ten additional reprecipitations by alcohol gave a substance (No. 59) of the following composition and rotation.

0 200 gm of substance containing 9 07 per cent moisture gave 0 0566 gm of $Mg_2P_2O_7$

0 100 gm of substance containing 9 07 per cent moisture gave 0 0644 gm of $BaSO_4$

0 200 gm of substance containing 9 07 per cent moisture used for Kjeldahl determination required 0 57 cc of 0 1 N HCl

	Calculated for $C_3H_7O_4PBa$ per cent	Found No 59 per cent
N	0 00	0 40
P	10 10	8 67
Ba	44 68	41 68

$$[\alpha]_D^{20} = \frac{+0\ 04 \times 100}{1 \times 18\ 19} = +0.22$$

All these salts agreed in property and appearance with the salts derived from the hydrolysis of lecithin by the same sort of purification. They all retained a distinctly yellow color which could not be wholly removed by bone-blacking the aqueous solution All specimens were very soluble in water and rotations were made on 20 per cent aqueous solutions.

The mother liquors from the purifications of No. 51 and its derivatives were concentrated in vacuum to a small bulk and precipitated with alcohol. The bulky precipitate was filtered and again dissolved in water. To this was added a 25 per cent aqueous solution of neutral lead acetate, and on standing lead glycerophosphoric acid settled out. This salt was filtered, suspended in water, and decomposed by hydrogen sulfide The precipitated lead sulfide was filtered and the filtrate concentrated to a small bulk The barium salt of glycerophosphoric acid was again isolated by adding barium hydroxide until slightly alkaline to litmus and precipitating with several volumes of alcohol. This

material was a glistening white, amorphous powder, identical in all characteristics with No. 63 obtained in an analogous manner from lecithin. Its composition and rotation were as follows. It contained no nitrogen.

0 1594 gm of substance containing 5 56 per cent moisture gave 0 0548 gm of $Mg_2P_2O_7$.

0 103 gm of substance containing 5.56 per cent moisture gave 0 0752 gm of $BaSO_4$

				Calculated for $C_3H_7O_6PBa$	Found No 64
				per cent	per cent
P	.	. ..		10 10	10 14
Ba .	. .		.	44 68	45 48

$$[\alpha]_D^{20} = \frac{-0\ 13 \times 100}{1 \times 18.97} = -0\ 69$$

Another hydrolysis of cephalin was made on 75 gm. of cephalin (No. 58) which were shaken for 16 hours at room temperature with $2\frac{1}{4}$ mols of barium hydroxide. The barium glycerophosphoric acid was isolated by the method used in the previous experiments, and after a preliminary precipitation with alcohol, was purified by conversion to the lead salt, and reprecipitation as the barium salt, according to the directions given under the foregoing experiment. After two additional precipitations by alcohol from its aqueous solution, the yield of this material (No. 68) was 5 gm. and its composition and rotation were the following.

0 101 gm. of substance dried under diminished pressure over sulfuric acid at temperature of xylene vapor yielded on combustion 0 0520 gm of CO_2, 0 0218 gm of H_2O, and 0 0684 gm of ash

0 200 gm of substance containing 7 84 per cent moisture gave 0 0626 gm of $Mg_2P_2O_7$

		Calculated for $C_3H_7O_6PBa$	Found No 68
		per cent	per cent
C. .		11 77	14 04
H.	.	2 29	2 41
P	.	10 10	9 92
Ba	.	44 68	39 12

$$[\alpha]_D^{20} = \frac{-0.9 \times 100}{1 \times 18\ 40} = -0.49$$

A second purification through the lead salt and one reprecipitation from alcohol gave a substance (No. 77) of the following composition and rotation.

0 099 gm of substance dried under diminished pressure over sulfuric acid in xylene bath gave on combustion 0 0484 gm of Mg_2CO_2, 0 0222 gm of H_2O, and 0 0692 gm. of ash

'0 300 gm of substance containing 9 00 per cent moisture gave 0.0956 gm. of $Mg_2P_2O_7$

	Calculated for $C_3H_7O_6PBa$ per cent	Found No 77 per cent
C	11 71	13 33
H	2 29	2 50
P.	10 10	9 76
Ba	44 68	41 36

$$[\alpha]_D^{20} = \frac{-0\ 12 \times 100}{1 \times 18\ 00} = -0\ 67$$

A third hydrolysis of cephalin was carried out in the same way on 50 gm. of cephalin (No. 135) and the purification of this material was exactly like that giving rise to No 77. The analysis and rotation of this material were as follows.

0 1011 gm of substance dried under diminished pressure over sulfuric acid in xylene bath, gave on combustion 0 044 gm. of CO_2, 0 0216 gm of H_2O, and 0 0724 gm of ash

0 200 gm. of substance containing 8 80 per cent moisture gave 0 0648 gm. of $Mg_2P_2O_7$

	Calculated for $C_3H_7O_6PBa$ per cent	Found No 145 per cent
C.	11 71	11 86
H	2 29	2 40
P.	10 10	9 89
Ba	44 68	43 88

$$[\alpha]_D^{20} = \frac{-0.13 \times 100}{1 \times 20\ 05} = -0\ 65$$

Like the barium glycerophosphates obtained from lecithin, these salts, after air-drying, contained variable amounts of water of crystallization approaching $1\frac{3}{4}$ mols, while after desiccation under diminished pressure for about 24 hours at room temperature, a more constant content of water was retained, corresponding to $C_3H_7O_6PBa\ H_2O$.

On complete desiccation over H_2SO_4 under diminished pressure at the temperature of zylene vapor, the following results were obtained.

0 1256 gm of No 59, previously air-dried, lost 0 0114 gm. of H_2O.
0.1088 " " " 77,   "   "   " 0 0098 " " H_2O
0 1104 " " " 78,   "   "   " 0 0093 " " H_2O.

	Calculated for $C_3H_7O_6PBa$ $1\frac{1}{2}H_2O$	No 59	Found No 77	No 78
	per cent	per cent	per cent	per cent
H_2O .. .	9 30	9 07	9 00	9 29

The substance dried at room temperature under diminished pressure lost on drying to constant weight at the temperature of xylene vapor as follows:

0 1164 gm. of No. 51 lost 0 0066 gm of H_2O
0.1116 " " " 64 " 0 0062 " " H_2O.

	Calculated for $C_3H_7O_6PBa$ H_2O	Found No. 51.	No 64
	per cent	per cent	per cent
H_2O	5 53	5 67	5 56

COMPARATIVE METABOLISM OF CERTAIN AROMATIC ACIDS.

III. FATE OF P-NITROPHENYLACETIC ACID IN THE ORGANISM OF FOWL, DOG, AND MAN.

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Many organic substances have been fed to animals to determine the process by which the animal body will dispose of them. If non-toxic, they may be wholly or at least in part oxidized and utilized for heat production.   If toxic, such compounds must be detoxicated and eliminated as rapidly as possible.   In the latter case we are able to study with a certain degree of precision what might be called the non-specific defense mechanism of the body.

We have already studied the method employed by the body when certain putrefactive compounds derived from phenylalanine and tyrosine are detoxicated and eliminated in the urine.   Oxidation and reduction play a large part in this process of detoxication, but often a point is reached where β-oxidation of the side chain is no longer possible, and in this case conjugation seems to be the method most often employed by the animal body   Thus benzoic acid no longer subject to oxidation in the organism is coupled with glycocoll and eliminated in the urine as hippuric acid.   Again, phenylacetic acid, which results from the action of bacteria on phenylalanine, cannot be oxidized by the tissues but is detoxicated by combining it with one of the shorter amino-acids   In this case the formation of the phenylacetic acid in the body of man and the lower animals seems to be the same type of reaction, but the process of detoxication is apparently more complicated in the animal than that taking place in the human body.

After feeding phenylacetic acid to animals such as dogs (1), rabbits (2), and monkeys (3), the acid appears in the urine com-

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bined with glycocoll, as phenaceturic acid; when ingested by human beings (4), phenylacetic acid is excreted in the urine in combination with glutamine, as phenylacetylglutamine. It has also been shown (5) that there is a difference in the processes of detoxication employed by man and animals after the feeding of *p*-hydroxybenzoic acid and *p*-hydroxyphenylacetic acid.

In the literature, one finds little concerning the metabolism of nitro compounds and particularly aromatic nitro derivatives. Sieber and Smirnow (6) fed large doses of *p*-nitrobenzaldehyde to a dog and recovered small amounts of *p*-nitrohippuric acid urea from the dog's urine.   Cohn (7) fed *p*-nitrobenzaldehyde to rabbits and isolated from their urine a compound made up of *p*-nitrobenzoic acid and *p*-acetylaminobenzoic acid.   Jaffe (8) fed "large amounts" (30 gm.) of *p*-nitrotoluene to a dog. He recovered some of the substance unchanged from the feces, some from the urine as *p*-nitrobenzoic acid, but most of it he found was eliminated in the urine as *p*-nitrohippuric acid urea.   Bertagnini (9) ingested small quantities of nitrobenzoic acid at frequent intervals, thus ingesting in all 80 gm. during a period of several days He was unable to prove the presence of either nitrobenzoic or nitrohippuric acid in the urine, but extracted from the urine an oily substance which gave nitrobenzoic acid and glycocoll on hydrolysis.

As there seemed to be no definite information in the literature regarding the fate of either the nitrobenzoic acids or nitrophenylacetic acids in the human body we decided to undertake some feeding experiments which eventually included not only man but the dog and fowl also   The compound used in these experiments was in each case *p*-nitrophenylacetic acid.

As oxidation of the side chain of phenylacetic acid seems impossible for either the animal or human body, there were several changes which this compound might undergo when ingested by a person.   It seemed most probable that the nitro group should remain untouched and that a combination with glutamine or glycocoll should be formed, and perhaps urea should also be added to the *p*-nitrophenylacetylglutamine or *p*-nitrophenacéturic acid thus formed.   On the other hand, a reduction of the nitro group was not impossible with a secondary acetylation of the amino group.

In the case of the dog it seemed most probable that p-nitrophenaceturic acid or p-nitrophenaceturic acid urea would be formed and eliminated in the urine.

The fowl seems to furnish, in most cases, large quantities of ornithine for the detoxication of foreign substances instead of glycocoll. Thus after feeding benzoic acid to chickens Jaffe (10) isolated from the urine a substance composed of 1 mol of ornithine and 2 mols of benzoic acid which he called ornithuric acid. Totani (11) after feeding phenylacetic acid to hens recovered a substance from the urine which he termed phenacetornithuric acid, and which was analogous to the compound isolated by Jaffe as it consisted of 2 mols of phenylacetic acid and 1 mol of ornithine.

Fate of p-Nitrophenylacetic Acid in the Human Organism.

The subject was a student of 70 kilos body weight and apparently in the best of health. The acid, being insoluble in water, could not be taken in the form of solution nor in capsules on account of bulk. A dose of 5 gm. of acid in crystalline form was accurately weighed out, taken into the mouth, and washed down with copious quantities of water   The acid possesses a very disagreeable taste not unlike pepper, with an after taste not unlike that of the copper salts

The urine was collected for 48 hours after each dose of the acid, each collection was carefully neutralized with sodium carbonate as soon as voided, then the entire amount slowly evaporated on a water bath at low temperature. The urine when evaporated to a thick syrup was cooled below room temperature and acidified with H_3PO_4 until it showed an acid reaction with Congo red This concentrated urine was placed in a continuous extracting apparatus and extracted for 5 hour periods with ether   The different ether extracts were placed in the ice box for a number of days to allow the formation of crystals   As no crystals appeared, the ether extracts were evaporated somewhat each day, then placed on ice over night   During this time no crystals appeared, so all the ether extracts were united and allowed to evaporate slowly at room temperature.   As dryness was reached, only a yellow oil appeared and no crystalline substance.   This

oily residue was dissolved in hot water, boiled with charcoal, filtered, and then allowed to cool   After a short time long yellow needles appeared in the solution and later the solution took on the form of a crystalline mass.   The crystals were removed by suction and recrystallized several times from hot water.   Dried at 80° they melted at 150–151.5.°   The melting point as well as the crystalline form showed the substance to be *p*-nitrophenylacetic acid which had been eliminated in the urine unchanged.

Analysis.—0.1533 gm. of the substance required 8 60 cc of 0.1 N sulfuric acid (Kjeldahl)

	Calculated for $C_8 H_7 N O_4$ per cent	Found per cent
N.	7 74	7 84

Only *p*-nitrophenylacetic acid crystallized out of the water solution of the ether extract, so it was evaporated to a small volume and repeatedly extracted with ether to remove the last traces of *p*-nitrophenylacetic acid.   Enough concentrated sulfuric acid was added to this water solution to produce a 30 per cent acid solution and the solution boiled under a reflux condenser for several hours.   The acid solution was cooled and repeatedly extracted with ether to remove any *p*-nitrophenylacetic acid which might be present.   The ether extracts were united, evaporated to dryness, and the residue dissolved in hot water.   No trace of *p*-nitrophenylacetic acid appeared at any stage in this extract so it seemed that no compound of *p*-nitrophenylacetic acid had been extracted from the urine by the ether.

It seemed reasonable to expect a compound of *p*-nitrophenylacetic acid with either glycocoll or glutamine so the evaporated urine was again extracted repeatedly with alcohol.   As *p*-nitrophenaceturic acid is quite insoluble in cold alcohol this substance should have crystallized out of the alcohol if present, but no crystals appeared.   The alcoholic extracts were united and evaporated, but only large amounts of urea and some hippuric acid crystals appeared.   The alcoholic extract was evaporated to dryness and dissolved in hot water, and the water solution acidified with sulfuric acid and boiled under a reflux condenser for several hours.   This acid solution was cooled and extracted with ether but again no *p*-nitrophenylacetic acid was found.   It appeared that no compound of *p*-nitrophenylacetic acid had been extracted

from the urine by the alcohol. The urine was further extracted with ethyl acetate and lastly with benzene following the same method as employed in the alcohol extraction, but no trace of the acid could be found. As a last resort a part of the evaporated urine was diluted with enough water to bring it into solution and strongly acidified with sulfuric acid. After boiling this for several hours it was cooled and extracted with ether to determine the amount of p-nitrophenylacetic acid which might have been split off from any compound existing in the urine. Again none of the acid appeared, so there certainly could have been no compound in the urine containing p-nitrophenylacetic acid.

After two 5 gm. doses of the acid had been ingested, a total of 6.87 gm. (68.70 per cent) of p-nitrophenylacetic acid was recovered from the urine as the uncombined acid.

It seems peculiar that this acid should be found free in the urine. While perhaps less toxic than phenylacetic acid, still some symptoms of intoxication such as nausea, headache, and diarrhea were experienced by the subject. It is certainly more irritating to the mucous membranes than benzoic acid, and benzoic acid taken in corresponding doses is almost quantitatively converted into hippuric acid.

Fate of p-Nitrophenylacetic Acid in the Organism of the Dog.

A dog of 32.70 kilos body weight was fed three doses of p-nitrophenylacetic acid   The acid crystals were pulverized and packed into large gelatin capsules. The capsules were inserted into large chunks of cooked meat which were thrown to the dog and swallowed without mastication. The first dose of the acid consisted of 5 gm.; a second dose of 5 gm. was fed 1 week later, followed by a third dose of 7 gm. 2 days later

The dog was kept in a large metabolism cage and the urine collected for 48 hours after each dose of the acid. The different portions of urine collected during this time were united and evaporated to a thick syrup, acidified with H_2SO_4, and extracted with ether for several hours in a continuous extracting apparatus. All portions of the ether used in this extraction were united and the ether was distilled off   The residue was dissolved in cold absolute ether, filtered, and the filtrate allowed to evaporate at room temperature. As no crystals appeared until the point of dryness was reached, the residue was redissolved in hot water,

boiled with charcoal, and allowed to stand in the ice box for 24 hours. Long yellow needles appeared in the solution and finally became almost a solid mass. The crystals were removed by suction and recrystallized from hot water. Dried at 80–90°, the crystals melted at 150–151.5°. The melting point as well as the characteristic solubility and crystalline structure of the substance showed it to be *p*-nitrophenylacetic acid which had been excreted in the urine unchanged   After a dose of 5 gm., 1.53 gm of the acid were recovered from the urine while, after a dose of 5 gm. followed after 48 hours by a dose of 7 gm , 5.32 gm. of the *p*-nitrophenylacetic acid were isolated from the dog's urine.

The evaporated urine was removed from the ether-extracting apparatus and placed in a large separatory funnel. It was then extracted several times with large volumes of hot alcohol   The alcoholic extracts were united and slowly evaporated to dryness. The residue was dissolved in hot water, boiled with charcoal, and filtered. The filtrate was slowly evaporated by stages until crystals appeared on cooling. This substance which seemed quite insoluble in water was thus easily separated from urea, hippuric acid, and other alcohol-soluble urinary constituents. Recrystallized from hot water, the substance formed extremely long hair-like needles. When dry, the substance melted at 172–173°. The melting point, as well as the solubility and crystalline form of the substance, showed it to be identical with the *p*-nitrophenaceturic acid prepared synthetically by Hotter (12).

0 2761 gm of substance required 11 45 cc of 0.1 N NaOH for neutralization

Calculated for $C_{10}H_{10}N_2O_5$. required 11.60 cc of 0 1 N NaOH.

2 gm of the substance were boiled for 2 hours with 30 per cent HCl, cooled, and the acid solution was extracted several times with ether   The ether extracts were evaporated to dryness and the residue was dissolved in hot water and boiled with charcoal After filtering, the filtrate was allowed to stand for some hours and crystals of *p*-nitrophenylacetic acid appeared. These crystals when dried melted at 150–151°. The amount of *p*-nitrophenaceturic acid isolated from the urine after a 5 gm. dose of *p*-nitrophenylacetic acid was 0 757 gm , while after a dose of 5 gm. followed by 7 gm of the acid, 2 873 gm of *p*-nitrophenaceturic acid were recovered.

The fate of p-nitrophenylacetic acid in the organism of the dog is similar to that of its homologue p-nitrobenzoic acid.

The p-nitrotoluene fed to dogs (8) was first converted into p-nitrobenzoic acid, followed by a secondary reaction in which most of this substance was converted into p-nitrohippuric acid urea. The greater part of the p-nitrophenylacetic acid was excreted in the urine uncombined while a part of it was conjugated with glycocoll and excreted as p-nitrophenaceturic acid. None of the latter compound, however, was found in combination with urea. After feeding 5 gm. of the p-nitrophenylacetic acid, 1.53 gm. (30.60 per cent) were excreted in the urine unchanged, while only 0 757 gm. (15.14 per cent) was excreted as the glycocoll compound. Thus a total of only 2 287 gm. (45.64 per cent) of the substance were recovered from the urine   After feeding 5 gm. followed by 7 gm of the substance, 5 32 gm (44.35 per cent) were isolated from the urine as the free acid and 2.873 gm. were obtained from the urine as the glycocoll compound (p-nitro-phenaceturic acid). Therefore after a total of 12 gm had been fed, 61.47 per cent of the substance was recovered

Fate of p-Nitrophenylacetic Acid in the Organism of the Fowl.

For this work a hen of 2.18 kilos body weight was selected. The acid was administered by means of a funnel and soft rubber tube. In this case a solution of the sodium salt was used and washed down with large quantities of water. ˙The dose in each case amounted to 1 gm. of the acid   The substance proved quite toxic, as the hen refused to eat for some days after the first dose and showed marked signs of depression. The hen was kept in a metabolism cage and all excreta saved for 48 hours after each dose. The excreta, which were always very hard and dry, were stirred into a paste after the addition of sufficient amounts of water. To this mass dilute sulfuric acid was added until the reaction was strongly acid to Congo red. It was then immediately extracted with ethyl acetate alcohol (10 : 1) mixture in a continuous extracting apparatus   The first and second extracts were evaporated to one-fifth the original volume *in vacuo* at 40°, then placed on ice for 48 hours   This process was repeated until the extracts became very concentrated but no crystals appeared. After complete evaporation, there remained a black tar-like mass. This substance seemed to be entirely insoluble in cold water but

slightly soluble in hot water   On cooling the hot water solution, it became a milky emulsion and under the microscope was found to contain fine droplets of an oil-like substance held in suspension. On cooling to room temperature the liquid became clear with a light yellow, oily deposit on the sides of the receptacle   There appeared to be two different substances present, one an oil-like substance and a second black gum-like substance which was unaffected by water. The water was poured off and both substances were dried at 40°. The mixture was extracted for 12 hours with ether in a continuous extracting apparatus. Most of the black gummy substance remained in the extracting apparatus but changed in color to a dull gray. The yellow substance dissolved easily in ether. The ether extract was filtered and allowed to evaporate at room temperature, leaving a yellow crystalline deposit. This crystalline mass was dissolved in hot water and slowly‿cooled. Long yellow crystals appeared in the water solution. The crystals were removed and dried at 80°. The melting point was between 149–150°, so the substance was apparently *p*-nitrophenylacetic acid.

0 2018 gm of the substance required 11 10 cc. of 0 1 N sulfuric acid (Kjeldahl)

	Calculated for $C_8 H_7 N O_4$ per cent	Found. per cent
N	7 74	7.70

The residue from the ether extract was dissolved in warm alcohol and allowed to cool; as no crystals appeared, ether was added until the solution became turbid   The flask was tightly stoppered and placed in the ice box for 24 hours. A fine deposit of microscopic crystals was found on the bottom of the flask so these were filtered off and more ether was added. By repeating this process, enough of the material was gathered for further work. This substance appeared to be insoluble in water but easily soluble in alkaline solutions. The substance was dissolved in barium hydroxide and the excess of barium removed by passing CO_2 through the solution and filtering off the barium carbonate. The filtrate containing the barium salt of the substance was evaporated by stages to one-third its original volume. After standing in the ice box for several days, large leaf-like crystals appeared on the surface of the liquid and formed a solid crust. The potassium and sodium

salts of the acid were formed from the barium salt by adding a solution of sodium and potassium sulfate to the solution of the barium salt   These salts were found to be too hydroscopic for analysis.   The water solutions of all the salts formed were found to be dextrorotatory.

To prepare the pure substance a solution of the barium salt was acidified with H_2SO_4 and extracted with alcohol.   The alcohol solution was concentrated and a few drops of ether were added until the solution became turbid.   After standing for several days in the ice box, short, thick, irregular needles appeared in the solu- tion.· These were recrystallized from an alcohol ether mixture and dried *in vacuo*.   The dried substance melted at 184–185°.

The analysis gave the following results.

0 1321 gm. of the substance gave 0 2662 gm. of CO_2 and 0 0576 gm. of H_2O.

0 2371 gm of the substance required 20 80 cc of 0.1 N sulfuric acid (Kjeldahl)

Calculated for $C_{21} H_{22} N_4 O_8$ C = 54.99 per cent, H = 4 84 per cent, N = 12.22 per cent

Found   C = 54 95 per cent, H = 4 99 per cent, and N = 12 30 per cent.

0 1191 gm of the substance required 2.70 cc of 0 1 N sodium hydroxide for neutralization   Calculated for $C_{21} H_{22} N_4 O_8$. 2.60 cc of 0.1 N NaOH were required

A weighed amount of the substance was boiled for 3 hours with a 30 per cent solution of HCl, cooled, and extracted with ether. The ether extracts were evaporated to dryness and the residue recrystallized from hot water.   The resulting yellow crystals when dried melted at 148.5–150°.

0 1007 gm of the substance required 5 45 cc of 0 1 N sulfuric acid (Kjeldahl)

Calculated for $C_8 H_7 NO_4$ N = 7 74 per cent

Found N = 7.58 per cent

This substance was *p*-nitrophenylacetic acid.   The acid solution remaining after the ether extraction was evaporated *in vacuo* to drive off the excess of HCl.   The residue was dissolved in a small amount of water and made alkaline with sodium carbonate.   To this alkaline' solution benzoyl chloride was added in small amounts at frequent intervals.   During the entire period the solution was kept alkaline, shaken vigorously after each addition of benzoyl

chloride, and cooled as often as necessary   After 45 minutes the solution was removed from the separatory funnel and acidified with HCl.   The acidified solution was extracted several times with ether to remove all benzoic acid formed.   After standing for some time a mass of leaf-like crystals appeared.   These crystals were washed chlorine-free and dissolved in warm alcohol.   By cooling the solution leaf-like crystals of ornithuric acid appeared.   The substance was pure ornithuric acid as was shown by the melting point of 184–185° and by the following analysis.

0 1262 gm. of the substance required 7 55 cc of 0 1 N sulfuric acid (Kjeldahl).

0 1136 gm of the substance gave 0 2801 gm of CO_2 and 0 0624 gm of H_2O.

Calculated for $C_{19} H_{20} N_2 O_4$ · C = 67 02 per cent, H = 5 92 per cent, and N = 8 23 per cent

Found   C = 67 23 per cent, H = 6 13 per cent, and N = 8 37 per cent.

This compound was identical with the ornithuric acid prepared by Jaffe (13), thus proving ornithine as the other constituent of the compound isolated from the excreta of the hen.

This compound must consist of 1 mol of ornithine and 2 mols of *p*-nitrophenylacetic acid and therefore has the following formula..
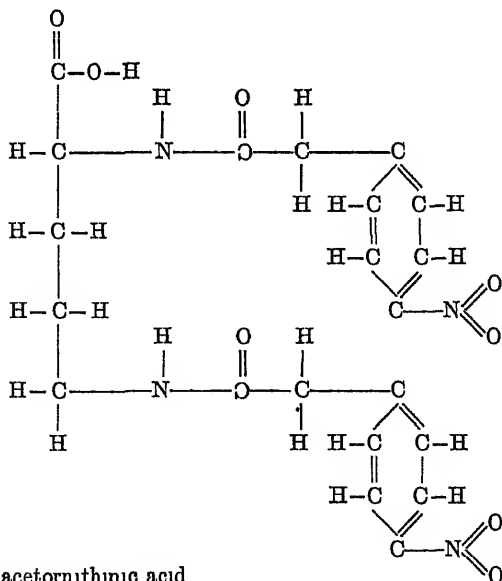
P-nitrophenacetornithinic acid

The combination of p-nitrophenylacetic acid with ornithine is quite in line with the results obtained by other investigators in experiments where fowls were used. In this case as in the experiments performed on the human being and on the dog no reduction or alteration of the nitro group was found in any case, as was reported by Cohn (7) after feeding rabbits p-nitrobenzaldehyde.

CONCLUSION.

1. p-Nitrophenylacetic acid was ingested by a man in 5 gm. doses. 68.70 per cent of the acid was recovered from the urine in an uncombined state. No compound of the acid was found in the urine.

2. p-Nitrophenylacetic acid was fed to a dog in 5 and 7 gm. doses. 61.47 per cent of the acid was isolated from the urine. Of this amount 44.35 per cent was in the uncombined state while 17.12 per cent was combined with glycocoll and excreted as p-nitrophenaceturic acid.

3. p-Nitrophenylacetic acid was fed to a hen in 1 gm doses. Some of the acid was excreted uncombined but the greater portion was conjugated with ornithine, and excreted as p-nitrophenacetornithinic acid. This acid which had not been previously prepared was isolated, analyzed, and its structure determined. The barium, potassium, and sodium salts of the compound were prepared and found to be dextrorotatory.

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STUDIES OF BLOOD REGENERATION.*

I. EFFECT OF HEMORRHAGE ON ALKALINE RESERVE.

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INTRODUCTION.

With the exception of Milroy's[1] work, no systematic attempt has been made, as far as the writer has been able to ascertain, to follow chemically the changes in alkaline reserve during the period immediately following hemorrhage. Since the Van Slyke[2] method has been devised, such a study has been greatly facilitated. Not only can more extensive data be obtained by the use of this method, due to its simplicity, but also the results so obtained have greater significance due to the fact that changes can be detected too small to influence the hydrogen ion concentration which Milroy determined.

The hemorrhages which Milroy[1] studied with cats and dogs were large (approximately one-third the total volume of blood). In all cases he found a distinct rise in the hydrogen ion concentration of the plasma, determined by the gas chain method; *i.e.*, a loss of reserve alkali. This change was most apparent in the cases in which the plasma was subjected to high concentrations of CO_2. Samples of blood drawn at intervals of 15 and 45 minutes after hemorrhage showed these distinct changes in reaction. Milroy concluded that there must have been rapid compensatory passage of the tissue fluids into the circulation, the fluid which first entered the circulation being extremely poor in reserve alkali. The greatest increase in hydrogen ion concentration was found after the 15 minute interval.

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[1] Milroy, T H , *J Physiol* , 1917, li, 259

[2] Van Slyke, D D , *J Biol Chem* , 1917, xxx, 347.

Since hemorrhage uncomplicated by other conditions such as shock and anesthesia is rarely encountered clinically many studies of these conditions are closely interwoven. Cannon and his co-workers[3] have studied clinically many cases of primary and secondary wound shock, hemorrhage, etc., with and without other complications, in a casualty clearing station near the front line trenches in France   In the uncomplicated hemorrhage cases, an examination of the blood disclosed lowered alkaline reserve.   In cases of acidosis and low blood pressure due to shock, hemorrhage, or infection with the gas bacillus, the pulse was rapid, but did not vary with the degree of acidosis   As the acidosis became more extreme, the respiratory rate increased, fatal cases showing true "air hunger" before death   As regards the relative reduction in alkaline reserve by the conditions of shock, hemorrhage, and infection with gas bacillus, Cannon's figures indicate the probability that uncomplicated hemorrhage is not followed by so great a reduction of alkaline reserve as is shock and infection with gas bacillus when the blood pressures are equally reduced

Penfield[4] observed that acidosis developed gradually when the blood pressures of etherized dogs were brought to a low level by progressive hemorrhage, about 100 per cent of the total blood volume (calculated as 5 per cent of body weight) being removed After varying lengths of time, the pressure was raised by infusion of different solutions.   With one exception, death occurred only in those animals whose alkaline reserve was low.

Morriss[5] studied the effect of ether anesthesia on the alkaline reserve, using Van Slyke's method   He found a reduction in the reserve alkali in every case, but this reduction was not dependent upon the duration of ether anesthesia or upon the extent of the operation.   He also found that the alkaline reserve might decrease notably when the patient lost relatively little blood, and on the other hand, might not change greatly when conspicuous hemorrhage occurred.   He concluded that a notable drop might occur in the carbon dioxide-combining power of the plasma during the first half hour of anesthesia.

[3] Cannon, W B , Fraser, J , and Hooper, A N , J Am Med Assn , 1918, lxx, 526   Cannon, W B , ibid , 531

[4] Penfield, W G , Am J Physiol , 1919, xlviii, 121

[5] Morriss, W H , J Am Med Assn , 1917, lxviii, 1391

The Reaction Regulator Mechanism.

Dependent upon the proper hydrogen ion concentration of the blood are such vital processes as the control of the respiratory and vasomotor centers, enzyme action, the swelling of colloids, and cellular oxidation. Fortunately the organism has a protective mechanism which guards against the acids which are constantly formed in the animal organism by the normal metabolism of its tissue and the foods ingested. This mechanism may be regarded as consisting of several factors; first, the function of the buffer salts, second, the ability of the organism to excrete the weak acids· (carbonic and phosphoric acids) set free by stronger acids; and third, the ability of the organism to produce ammonia at the expense of urea.

Van Slyke's method for the determination of alkaline reserve is based upon the principle that the power of the organism to protect itself against the encroachment of acids is proportional to the bicarbonate content of the arterial blood. In actual practice Van Slyke analyzes the plasma obtained from centrifugation of venous blood and reports the "alkaline reserve" in terms of volumes per cent of carbon dioxide chemically bound by 100 cc. of plasma. The possibility that HCl may pass from plasma to cells or *vice versa*, depending on the CO_2 tension, makes the precaution necessary that the blood should always be at definite CO_2 tension when centrifuged, as well as when analyzed. Van Slyke[2] and Austin and Jonas[6] carefully studied the effect on alkaline reserve of variations in the technique used in collecting the blood, and preparing the plasma for analysis.

The immediate cause and opportunity for this investigation were furnished by the plant for making serum to be used in vaccination against hog cholera, which is operated in connection with the College of Agriculture of the University of Wisconsin. In this plant large numbers of pigs are hyperimmunized against hog cholera. The animals are subsequently bled, the blood is defibrinized, and the serum used commercially. In this process, a certain routine, found by experience to be satisfactory and economical, has been established. Approximately 10 days after the animals have been hyperimmunized they are subjected to

[6] Austin, J H , and Jonas, L , *Am J Med Sc* , 1917, cliii, 81

tail bleeding, if they have not lost in weight. After an interval
of 7 days the pig is subjected to a second tail bleeding if it has
not lost in weight, and after 7 more days the pig is slaughtered.

In preliminary experiments, the blood was treated by what
will hereafter be referred to in this paper as the "open vessel
method" or Method I   The blood was allowed to flow directly
from the tail into a small open Erlenmeyer flask which contained
sufficient potassium oxalate to prevent clotting. Under the con-
ditions of the experiment, the blood spurted rapidly from the
artery directly into the flask, and so came into minimum contact
with the tissues. No needle or other apparatus was required
in collecting the sample. The flask was then rotated once or
twice to insure the oxalate being dissolved in the blood, and was
then carried to the laboratory for centrifugation with as little
agitation as possible. The interval between collection and
centrifugation was approximately ½ hour.

Standardization of Methods.

In order to discover the effect of this treatment on the alkaline
reserve value, the following experiments were performed. Ani-
mals were kept at the laboratory in cages, and samples of blood
were collected and treated under different conditions. In the
first instance a stream of blood was allowed to spurt directly from
the tail artery into a paraffined Erlenmeyer flask containing a
small amount of potassium oxalate. The blood was centrifuged
and the plasma analyzed by means of Van Slyke's technique.

This "open vessel method," when the blood was centrifuged
and the plasma analyzed immediately, was very nearly compar-
able with Van Slyke's routine technique when he used a McRae
needle for collection. By the open vessel method, it is true, the
blood necessarily falls twice for a short distance through the air.
This fact would seem to double the possibility for loss of carbon
dioxide. So much less agitation was necessary, however, to
insure thorough mixing of the oxalate with the blood in a recep-
tacle like an Erlenmeyer flask that it seemed preferable to make .
the collections in such a flask rather than in a centrifuge tube.

This was particularly true in cases where the blood clotted rapidly, as after repeated hemorrhage. Because of the necessity, when working with a pig, of taking the blood samples from a cut tail, it was impossible to collect samples by Van Slyke's paraffin oil method; i.e., without loss of some carbon dioxide.

Open Vessel Method —Bloods collected by the open vessel method in the presence of sufficient potassium oxalate to prevent clotting were treated as follows

I-a-1 —The blood was poured directly into centrifuge tubes, centrifuged, and the plasma analyzed at once (Curve I, Fig 1)

I-a-2 —The blood was centrifuged as described under I-a-1. The clear plasma was pipetted into a paraffined weighing bottle, preserved in a refrigerator for 48 hours, and was then analyzed (Curve II, Fig 1)

I-b —The blood was allowed to remain in the Erlenmeyer flask in which it was collected for $\frac{1}{2}$ hour It was then centrifuged and the plasma analyzed immediately (Curve III, Fig. 1)

I-c —The technique was the same as I-b, the time before centrifugation being extended to 2 hours (Curve IV, Fig 1)

In the second instance an effort was made to approximate Van Slyke's paraffin oil method by allowing the blood to flow directly from the tail artery into a paraffined separatory funnel which had been previously filled with alveolar air. This technique was called the "separatory funnel method" or Method II. Duplicate collections of the blood analyzed by the open vessel method were made by the separatory funnel method and the samples so obtained were treated in various manners. Since the separatory funnel method was more laborious, and the results obtained were not duplicated so easily, this method proved to have no advantage over Method I, and was consequently abandoned For the sake of brevity, the analyses by this method are not reported here.

Table I gives the results of the preliminary analyses In all cases the figures represent the volumes per cent of CO_2 obtained from 1 cc. of plasma, and are the average of closely agreeing duplicates After the first three series, all collections were made in paraffined vessels.

All the samples reported in the same vertical column of Table I were taken from the same animal, one sample immediately following another, and were then treated in the various manners described The blood for the first five series of determinations was obtained from a female, for the second four series from a male.

Each weighed approximately 200 pounds. The extreme variations in the alkaline reserve values of the blood from the same animal on different days will be discussed in detail later.

The column headed "average difference" was computed as follows. The first method of determination (the open vessel method, centrifuged and analyzed immediately) was arbitrarily selected as the standard. If subsequent determinations were lower, the difference was indicated by a negative sign, and if

TABLE I

Effect of Various Methods on Alkaline Reserve Values.

Method	Alkaline reserve of blood from Pig 1					Alkaline reserve of blood from Pig 2				Average difference
	June 27	June 27	June 28	July 2	July 3.	July 5.	July 5.	July 6	July 8	
	a m	p m	a m	a m	a m	a. m	p m	a m	a m	
I-*a*-1. Centrifuged and analyzed immediately	49 4	56 7	25 8	53 1	58 4	51 3	29 1	48 3	54 0	±0
I-*a*-2 Centrifuged immediately, analyzed after 2 days	46 4	53 5	29 5	52 8	59 0	50 8	27 9	48 4		—4 5
I-*b* Centrifuged after ½ hour; then analyzed at once	36 7	48 8	27 7	51 9	56 5	49 7	27 2	45 0	52 6	—3 3
I-*c*. Centrifuged after 2 hours, then analyzed at once		49 2	29 6	51 9	54 1	46 5	26 3	44 2	50 3	—3.1

higher, by a positive sign. It will be seen that the average fall in alkaline reserve after intervals of ½ and 2 hours respectively is almost identical, and the maximum variation in the entire series between these two values is 3.2 volumes per cent.

The results obtained by the open vessel method are expressed graphically in Fig. 1. The alkaline reserve values are plotted on the vertical axis, and values in the same series (that is on the bloods collected at the same time, but subjected to different techniques) are given the same position on the horizontal axis.

Consequently, consistent results among the different techniques should yield parallel lines. That the lines to a certain extent approximate the parallel is an indication not only that the results can, within limits, be duplicated, but also that the time element is not an all-important factor. In other words, samples analyzed $\frac{1}{2}$ hour or 2 hours after collection may not give identical values with the same samples analyzed immediately, but at least, corresponding time intervals yield samples that are, within limits, comparable. There is not extreme variation between short intervals, as, for instance, the $\frac{1}{2}$ and 2 hour intervals.
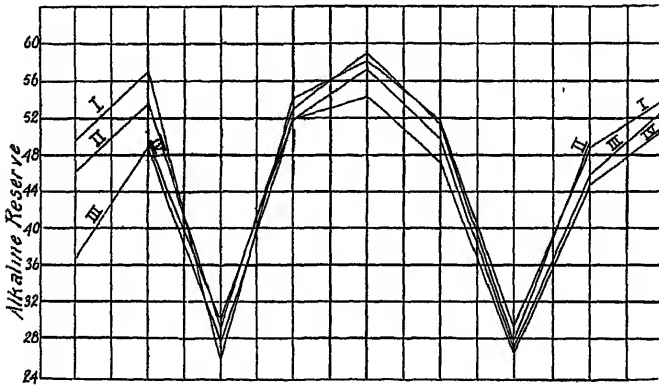
Fig 1 Open vessel method   Illustrates the effect of different preliminary treatment on the alkaline reserve values of the same bloods. Alkaline reserve values are plotted on the ordinates and values obtained from the same collections, but analyzed by various techniques, are given the same position on the abscissæ   The general parallelism of the lines indicates that the results can be duplicated and that the various treatments yield consistent results.

Another attempt was made to find a technique which would be comparable with Van Slyke's method in which there was no loss of CO_2 during collection. Austin and Jonas[6] found that when they brought the whole blood into equilibrium with alveolar air immediately before centrifugation, their results were slightly higher than results obtained by Van Slyke's method, but were consistent with them. Since the details of Austin and Jonas' procedure could not be found, the following procedure was adopted. This technique will subsequently be referred to as the "alveolar air method" or Method III.

The blood was allowed to flow directly from the tail blood vessels into a paraffined separatory funnel of 300 cc. capacity, into which sufficient potassium oxalate had been introduced to prevent clotting.   The funnel was then turned upside down once or twice to insure the oxalate being mixed with the blood.   Just before the blood was centrifuged, alveolar air was blown into the funnel, and the funnel was then rotated end over end at the rate of one complete revolution per second for 60 seconds   Alveolar air was again blown into the funnel, and the process was repeated. In all, three such aspirations were made.   Theoretically, if the composition of the alveolar air in the funnel were 6 per cent CO_2, then, during the three aspirations, 54 cc of CO_2, measured at the same temperature and pressure, would have been introduced. The average difference (as will be shown later) between simultaneous determinations by Methods I and III is approximately 10 volumes per cent, or 0.10 cc. of CO_2 for every 1.0 cc. of plasma. It is not known how much CO_2 is absorbed by the corpuscles under these conditions.   Furthermore, a liquid and a gas are in equilibrium not when their percentage compositions are the same but when their partial pressures are equal, i e., when the pressure of the gas on the liquid is equal to the tendency of the gas to leave the surface of the liquid   Since the actual mass of CO_2 absorbed by the blood from the alveolar air was such a small proportion of the mass of CO_2 actually introduced, and since the alveolar air was introduced in three separate portions, and each time was allowed to come into intimate diffusion relations with the blood, it seemed highly probable that the blood had absorbed as much CO_2 as it could be made to hold under these conditions, and that this technique was comparable to that of Austin and Jonas in which they brought the blood into equilibrium with alveolar air before centrifuging   The whole blood was then drawn off from the separatory funnel into a centrifuge tube under paraffin oil and was centrifuged under a layer of the oil.   A great many determinations of alkaline reserve were made from samples of blood which were simultaneously collected, centrifuged, and analyzed, one sample being treated in the manner described for Method I and another in the manner described for Method III. In Tables II, III, and IV and Figs. 2 to 8 will be found the records of the determinations so made.   In some cases (Figs 2,

3, and 4, and Table II) a definite effort was made to ascertain the effect on the alkaline reserve values of allowing the blood to stand varying lengths of time before being centrifuged. The object of this experiment was to standardize the methods which might be used in subsequent experiments. In this work it was not the absolute alkaline reserve values of the blood as it existed

TABLE II

Effect of Time before Centrifugation on Alkaline Reserve Values by Various Methods

Animal No	Time after collection before centrifugation	Alkaline reserve		Temperature	Remarks
		Method I	Method III		
	min			°*C*	
3	70	54 3	60 5	32	See Fig 2.
	70	53 7	59 6		
	185.	51 7	55 6		
	185	50 1	56 9		
	360	50 6	53 5		
	360	50 1	53 5		
4	55	48 1	60 6	26	See Fig 3.
	85	48 1	58 6		
	115	48 1	60 0		
	145	48 6	58 1		
	175	48 2	58 3		
	305	48 0	56 4		
5	75	26 7	41 4	22	See Fig 4
	105	26 4	40 9		
	135	26 2	39 7		
	165	25 9			
	195	26 7	25 9		

in the animal body which were sought, but the relative values before and after hemorrhage.

It was noticed early in the work that fairly constant differences existed between the values obtained by Methods I and III for different sets of samples collected and analyzed under the same conditions. For instance, on one day, the average difference of six sets of samples analyzed by Methods I and III was 4 9, the maximum variation from this average figure being ± 1.6. On

another day, the average difference between the two methods run on the same number of samples was 8 2, the maximum variation from this figure being 1.9.   Figs. 2 to 8 show that curves representing values obtained by the two methods for the most part run fairly parallel, each curve tending to afford evidence of the comparative accuracy of the data represented by the other. Furthermore, the curves plotted from data obtained by Method I are noticeably more regular, and hence are subject to fewer minor variations, than those plotted from data obtained by Method III. The degree of accuracy with which separate collections and centrifugations of samples could be duplicated by the two methods is indicated by the first series of data in Table II.   An average of
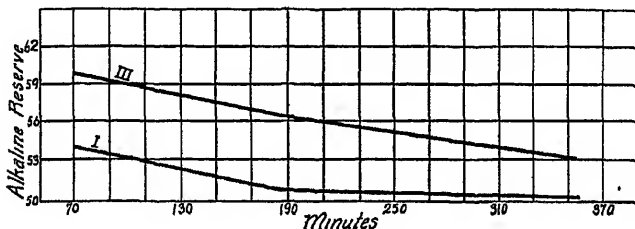
FIG 2. On Curves I and III are plotted the alkaline reserve values obtained from samples collected at the same time, but allowed to stand definite periods of time before being centrifuged. Curve I represents values obtained by the open vessel method; Curve III, by the alveolar air method. Duplicate collections as well as duplicate analyses were made in each case, and average figures used. Temperature 32°C.

these duplicates was used in plotting Fig. 2   Although, as will be explained presently, Method III more nearly approaches an accurate picture of the true alkaline reserve values, results obtained by Method I are more easily duplicated, and apparently bear a more or less definite relation to the alkaline reserve values of the blood as it exists in the animal body.

A partial explanation for the variation in the relative values obtained by the two methods was found in the variation in temperature at the time when the samples were centrifuged. It happened that the data expressed in Fig 2 were obtained on a summer day when the temperature was 32°C. The time in minutes after the samples were collected before they were centrifuged and

analyzed (or preserved in paraffined weighing bottles in an ice box) is plotted on the horizontal axis, the alkaline reserve values being plotted on the vertical axis. Although these data are very meager, they would seem to indicate that there was a small, though appreciable loss of CO_2 from the samples, even after the hour period, a point which was not illustrated in Fig. 1. To test further this possibility, two similar experiments were run, when the temperature was somewhat lower (Figs. 3 and 4). These results tend to uphold the conclusion that after the initial loss in CO_2 by a blood sample, which occurs during the first ½ hour after the blood is drawn, there is very little subsequent loss during the following 2
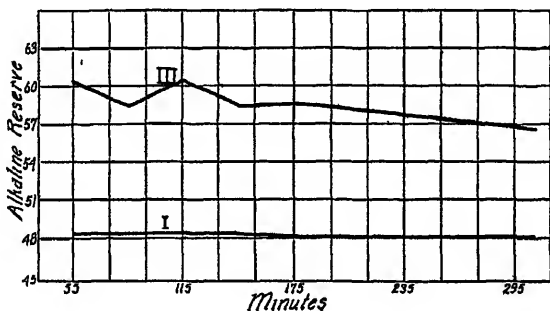
FIG. 3. Curve I represents alkaline reserve values obtained by the open vessel method on simultaneous collections which were allowed to stand varying lengths of time before centrifugation Curve III represents values obtained on duplicate collections, treated by the alveolar air method. Temperature 26°C In general, values obtained by Method III are higher than those obtained by Method I, but are consistent with them.

hours at ordinary temperatures; and that the values so obtained bear a more or less definite relation to those obtained when the blood is analyzed as soon as it is drawn. In only one case was a single value obtained which was entirely out of harmony with these ideas. This value is plotted at the last point in Curve III, Fig. 4. This plasma had been kept in an ice box for 4 days before it was analyzed. In general, the experience of the author entirely confirmed that of Van Slyke, inasmuch as plasma could be kept in an ice box for 7 days without appreciable loss in CO_2 content. The conclusion seems justifiable in this case that the plasma had undergone bacterial change or had become contaminated in some

other way.   This contention is supported by another experiment
Two 15 cc. samples of blood (the alkaline reserve values of dupli-
cate collections, by the open vessel method, were centrifuged and
analyzed immediately, and were 53.8 and 58 4 respectively) were
subjected for 15 minutes to the reduced pressure produced by a
powerful vacuum pump.   The blood was then centrifuged and
analyzed, these values being 38.7 and 39 6 respectively   In these
two cases, where the maximum loss of CO_2 might have been ex-
pected, the actual loss was 15.1 and 18 8 volumes per cent.   Doubt-
less, then, the drop of 15 8 referred to above was not due to
accidental loss of CO_2.
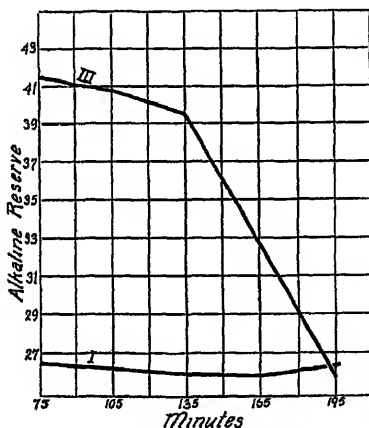
Fig. 4 Curve I represents values obtained by the open vessel method
and Curve III by the alveolar air method, as in case of Figs. 2 and 3   Tem-
perature 22°C   The plasma represented by the last point on Curve III
had undoubtedly undergone bacterial change

Although temperature is doubtless a contributing factor to the
relative differences found in the analysis of duplicate samples by
Methods I and III, examination of the curves indicates that, in
general, larger differences were obtained when samples having
comparatively high alkaline reserve values were analyzed.   Fig. 8
illustrates this point   When the lowest values were reached at
the 60 minute interval, the difference was 3 volumes per cent as
compared with an initial difference of 15   If one had two solutions
containing carbon dioxide in different proportions, and if one

manipulated these solutions in such a way that some carbon dioxide was lost, both solutions being treated in like manner, it seems reasonable to suppose that a larger mass of carbon dioxide would be lost by the solution containing the larger per cent of carbon dioxide. If one may reason by analogy, it seems logical that more carbon dioxide would be lost in the open vessel technique if the initial bicarbonate content were high than if it were low Since loss of carbon dioxide is guarded against by Method III, greater differences between the two methods would be apparent when the initial bicarbonate content was comparatively high

Influence of Hemorrhage on Alkaline Reserve.

The most surprising feature of the alkaline reserve values obtained in this preliminary work on methods was the fact that blood shed by the same animal varied tremendously on different days (Table I). The values recorded in the columns headed June 28 a.m and July 5 p.m were in each case obtained from the same animal as the values in the columns immediately preceding and following   The idea might be advanced that the drops in alkaline reserve were the result of loss of blood from a previous bleeding. This seems highly improbable, however, because in each bleeding in this series, not more than 100 cc. of blood were taken at a time Furthermore, there was no relationship between the frequency of bleeding and the alkaline reserve values. As the experiment proceeded, it became evident that the high values were invariably associated with the bleedings which were accomplished without any struggle on the part of the animal before or at the time when the blood was drawn. The two extremely low values referred to were associated with violent struggling, and the intermediate values represented corresponding degrees of struggle. This point is further borne out by subsequent experiments. The data in Table III, which are taken from a series of tail bleedings, illustrate this point. In this table, a lower alkaline reserve value at the end of the bleeding is shown by a positive sign in the column headed "loss in alkaline reserve during bleeding," and *vice versa*. It is evident that, in those cases where little or no struggle took place during bleeding, the alkaline reserve values of the last 10 cc. of the large bleeding did not differ greatly from the values of the

first 10 cc. This fact is emphasized here in order that it may be borne in mind in connection with the data obtained in sub-sequent bleeding experiments.

In a preliminary experiment an attempt was made to follow the alkaline reserve values of a pig daily. Because of the difficulty in obtaining samples, and because the work of Milroy and others pointed to the fact that the drop in alkaline reserve caused by hemorrhage occurs soon after the blood is shed, no further attempts

TABLE III

Typical Changes in Alkaline Reserve during the Process of Bleeding

Animal No	Weight	Blood drawn	Meth-od	Alkaline reserve		Loss in alkaline reserve during bleed-ing	Remarks
				1st 10 cc drawn	Last 10 cc drawn		
	lbs	cc					
21	289	1,400	I	47 3	43 9	+3 4	Quiet throughout, bled' freely.
21	289	1,400	III	55 9	56 9	−1 0	
7	245	1,000	I	50 4	51 2	−0 8	Quiet throughout; bled' slowly.
52	272	1,000	I	51 2	48 9	+2.3	Quiet throughout; bled slowly
18	210	1,300	I	61 6	59 7	+1 9	Quiet throughout; bled' freely.
18	210	1,300	III	69 2	68 1·	+1 1	
6	291	1,400	I	45 1	29.1	+16 0	·Quiet before bleeding; struggled violently dur-ing bleeding; bled free-ly
6	291	1,400	III	54 5	36 1	+18 4	

were made to collect daily samples. The procedure subsequently adopted was as follows. The animal was confined in a crate, and the tail was shaved. A small portion of the tail was then cut off, and the first few cc. of blood (about 5 cc.) were discarded. When the blood spurted from the artery in a good stream, two small samples (about 10 cc. each) were collected for analysis by Methods I and III. The results from these samples in each case were plotted as the first points on the curves. The cut tail was introduced into the vacuum flask and sufficient blood was drawn

to approximate 6 cc. per pound of body weight. Two more small samples were then taken as before, and the time of sampling was noted. The intervals between the samples at the beginning and at the end of the large bleeding indicated the duration of bleeding. From these data the approximate rate of bleeding could be calculated. Since the rate of bleeding varied consider-
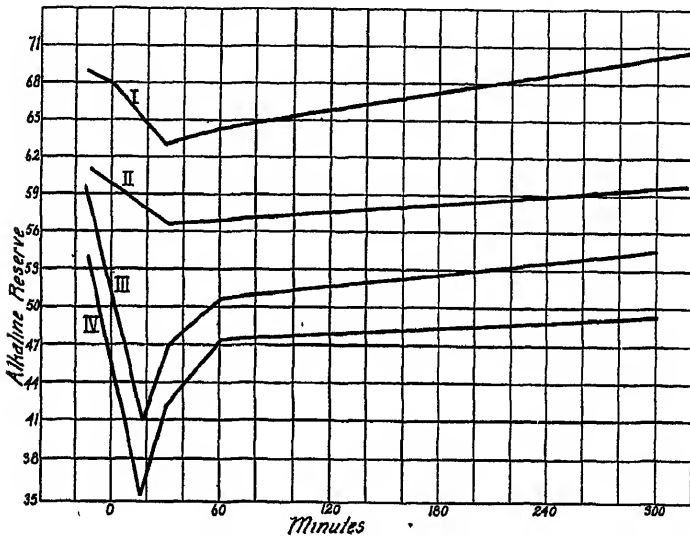
Fig 5 Curve I (alveolar air method) and Curve II (open vessel method) illustrate the drop in alkaline reserve following the first hemorrhage of Sow 20 The time (abscissæ) is expressed in minutes before or after the large hemorrhage was completed, i e, the first point on the curves represents the alkaline reserve values of the first blood shed, the second point (at 0) the values of the last 10 cc of the large bleeding Curve III (alveolar air method) and Curve IV (open vessel method) represent a second hemorrhage of the same animal a week later (Table IV).

ably at different times, all subsequent samples were timed from the minute at which the large sample had been completely drawn. The animal was left in the crate, and the samples were taken at the intervals desired. Loss of blood between samples was prevented by tightly winding a rubber band around the end of the tail. At the end of the hour, the pig was taken from the crate, put into a pen, and was not disturbed until another sample was

TABLE IV

Effect of Hemorrhage on Alkaline Reserve

Animal No	Description	Weight	Blood drawn	Time of sampling	Alkaline reserve		Remarks
		lbs	cc		Method I	Method III	
20	Not hyper-immune ♀	210.	1,260	First 10 cc of big bleeding	61 6	69 2	Bleeding lasted 8 min ; very quiet throughout, no difficulty with respiration (Fig 5)
				Last 10 " " "	59 7	68 1	
				30 min after end of "	56 5	63 8	
				60 " " "	57 4	64 9	
				317	80 6	70 7	
20 (7 days later)	Not hyper-immune ♀	234	1,400	First 10 cc. of big bleeding	54 3	60 5	Struggled just when samples were taken at end of big bleeding, struggled considerably during first ½ hr after bleeding, restless during second ½ hr , respiration normal throughout, necessary to cut the tail again to collect the last sample, bleeding lasted 14 min. (Fig 5)
				Last 10 " " "	46 6	50 8	
				15 min after end of "	35 3	41 0	
				30 " " "	42 3	47 0	
				60 " " "	47 6	50 9	
				300	49 5	55 1	
21	Hyper-immune ♀	299	1,333	First 10 cc of big bleeding		53 0	Bleeding lasted 8 min , struggled when samples were taken, otherwise quiet throughout, respiration normal (Fig 6)
				Last 10 " " "		47 0	
				15 min after end of "	37 9	44 5	
				30 " " "	33 7	37 7	
				60 " " "	30 9	39 0	
					31 0		
22	Hyper-immune. ♀	202	1,260	First 10 cc of big bleeding	48 1	60 6	Bleeding lasted 23 min , poor bleeder, tail was cut 3 times during big bleeding; fairly quiet throughout, except when the last samples were taken (Fig 7)
				Last 10 " " "	41 9	51 2	
				15 min after end of "	41 9	50 7	
				30 " " "	43 0	49 5	
				60 " " "	48 0	60 5	
				330	46 1	52 5	

23	216	1,300	First 10 cc of big bleeding	26 7	41 4	Bleeding lasted 37 min ; blood unus-
Hyper-immune ♂			Last 10 " " " " "	12 3	23 4	ually dark, struggled all the time
•			15 min after end of " " "	12 3	23 3	while in the crate (1½ hrs), all anal-
			30 " " " " " "	12 1	14 0	yses run in duplicate, animal sur-
			60 " " " " " "	2 0*	5 0*	vived (Fig 8)
			287	21 7	26 2	

* These values were obtained by plotting the values given in Van Slyke's tables and reading these values from the curve

taken in the same way at the end of an approximate 5 hour interval. In most cases it was a simple matter to obtain the 5 hour sample. Occasionally it became necessary to cut the tail again. In such cases the fact has been noted.

In the first experiment of this kind, samples were collected for analysis by both methods when 30 and 60 minutes had elapsed after the big sample had been drawn. In this case, as is shown by Curves I and II, Fig. 5, the drop in alkaline reserve was comparatively small, the lowest point being reached at the 30 minute interval. The 5 hour sample showed that the alkaline reserve
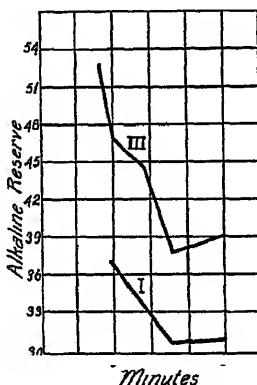
FIG. 6 Curve III (alveolar air method) and Curve I (open vessel method) illustrate the performance of Sow 21, a hyperimmune, during a second hemorrhage (Table IV).

was very nearly at its original value. Since the drop in alkaline reserve was so small (about 5 volumes per cent) it seemed likely that the low point on the curve had been missed. In the subsequent experiments, samples were taken 15 minutes after the end of the large bleeding also.

Curves III and IV, Fig. 5, show the performance of the same animal 7 days later, the total quantity of blood drawn being slightly larger than that taken the previous week. In this case, the alkaline reserve was consistently lower throughout. The drop in alkaline reserve on that day, due, presumably, to loss of blood, was much greater; the lowest point being reached at the end of 15 minutes.

Considerable data of this nature were accumulated, without any attempt at further analysis of these samples. The data are summarized in Table IV, and expressed graphically in Figs. 5, 6, 7, and 8. Further information concerning the drop in alkaline reserve associated with loss of blood is furnished by subsequent more elaborate experiments.

These experiments were of particular interest inasmuch as they indicated that there was great variation in the drop in alkaline reserve due to hemorrhage. It is a well recognized fact that after loss of blood, the blood volume is rapidly restored, and an efficient
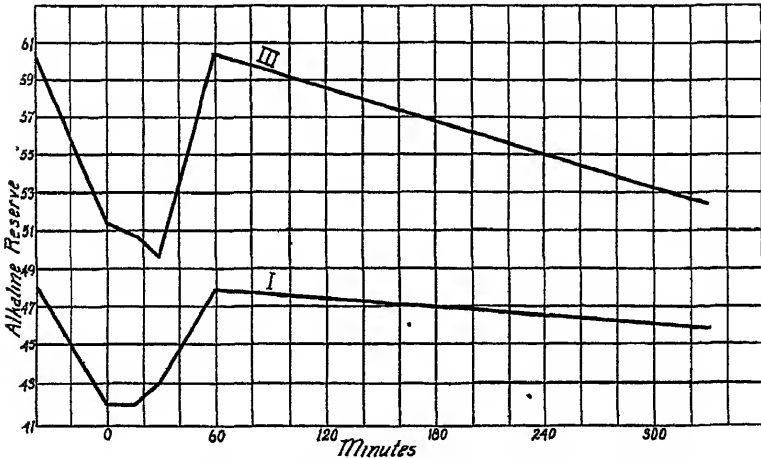
FIG 7. Curve III (alveolar air method) and Curve I (open vessel method) illustrate the effect of a first hemorrhage on Sow 22 (Table IV).

blood pressure consequently maintained, by a dilution of the blood with the tissue fluids. Consequently, a drop in the total nitrogen content of the blood might be expected.

Two pigs (females, Nos. 7 and 8), which were not hyperimmune, were bled in the usual manner, the alkaline reserve values (obtained by the open vessel method) being recorded in Tables V and VI, and plotted in Figs. 9 and 10. Three successive bleedings were made on these two animals, at weekly intervals. The total nitrogen of the blood was determined by the Kjeldahl method, the samples being weighed in all cases. The results are averages of closely agreeing triplicate determinations. The usual drop in

percentage of nitrogen in 5 hours, due to hemorrhage of this magnitude, was 0 3 to 0 4 per cent   No explanation is advanced for the unusual drop of 1.01 per cent found in the second bleeding (Table V).   More data on this point will be presented later. This experiment illustrates the individual reaction of animals subjected to the same treatment.   The animals described in Tables V and VI were similar in size, weight, and sex, but one
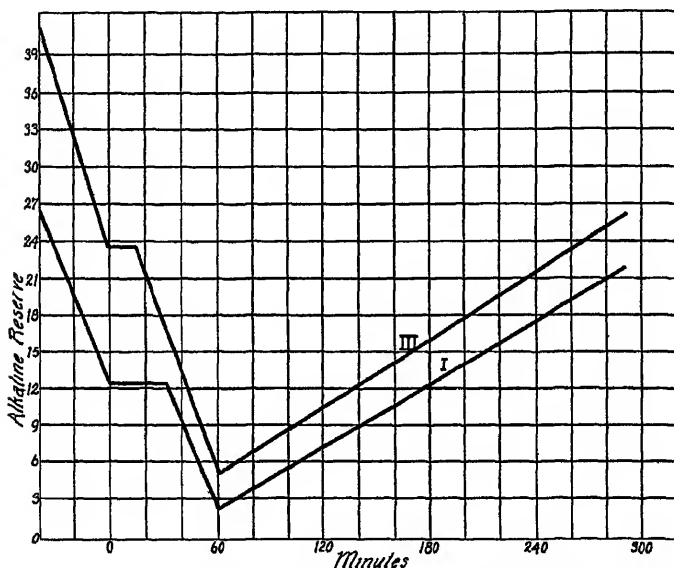
Fig. 8. Curve III (alveolar air method) and Curve I (open vessel method) illustrate the effect of a first hemorrhage on No 23, a black male   The alkaline reserve values reached the lowest point encountered in this work This experiment shows the combined effect of violent struggle plus hemorrhage in lowering alkaline reserve (Table IV)

suffered a greater loss in reserve alkali on bleeding than the other. No. 8 (Table VI) developed an acidosis, due to violent struggle and loss of blood, which was less severe, in terms of actual alkaline reserve values, than that developed by No. 7 (Table V, second bleeding) when she was quiet throughout.   The drop in alkaline reserve was the same in both cases.   It is evident from data gathered by the repeated bleeding of the same animal that the loss of reserve alkali immediately following hemorrhage was not

TABLE V

Effect of Repeated Hemorrhage on Blood of Pig 7

Date	Weight	Blood drawn	Time of sampling	Alkalinereserve		Total nitrogen		Remarks
				Volumes per cent	Maximum drop	Per cent	Drop	
	lbs	cc						
1918 Oct 4 1st bleeding	270	1,625	First 10 cc of big bleeding	47 3		3 16		Fairly quiet throughout (Fig 9, Curve I)
			Last 10 " " " "	44 5				
			15 mn after end of "	44 5				
			30 " " " "	42 6				
			60 " " " "	39 0	8 3			
			325	44 6				
Oct 11 2nd bleeding.	280	1,680	First 10 cc of big bleeding.	42 2		2 85		Fairly quiet throughout (Fig. 9, Curve II)
			Last 10 " " " "	43 0		2 90	0 31	
			15 mn. after end of "	34 6				
			30 " " " "	26 6	16 4			
			60 " " " "	34 0				
			297	36 4				
Oct 18 3rd bleeding.	276	1,650	First 10 cc. of big bleeding	63 0		1 89	1 01	Fairly quiet throughout. (Fig 9, Curve III)
			Last 10 " " " "	62.5		2 72		
			15 mn after end of "	54 8				
			30 " " " "	51 2	11 8			
			60 " " " "	54 6				
			300	54 3		2 44	0 28	

TABLE VI.

Effect of Repeated Hemorrhage on Blood of Pig 8.

Date	Weight	Blood drawn	Time of sampling	Alkali reserve		Total nitrogen		Remarks
				Volumes per cent	Maximum drop	Per cent	Drop	
1918	*lbs*	*cc*						
Oct. 4 1st bleeding	270	1,625	First 10 cc of big bleeding	56 7		2 89		Struggled considerably (Fig. 10, Curve I)
			Last 10 " " "	47 3				
			15 min after end of "	47 3				
			30 " " " "	40 4	16 3			
			60 " " " "	44 6				
			288	52 5				
Oct. 11 2nd bleeding.	298	1,790	First 10 cc of big bleeding	47 5		2 59		Uneasy during bleeding, then fairly quiet (Fig 10, Curve II)
			Last 10 " " "	44 8		2 74	0 30	
			15 min after end of "	43 9				
			30 " " " "	46 1	3 6			
			60 " " " "					
			260	46 5				
Oct. 18 3rd bleeding	276	1,650	First 10 cc of big bleeding	61 1		2 33	0 41	Fairly quiet throughout (Fig 10, Curve III)
			Last 10 " " "	61 1		2 78		
			15 min after end of "	56 4	4 7			
			30 " " " "	58.3				
			60 " " " "	58 3				
			283	57 0		2 31	0 47	
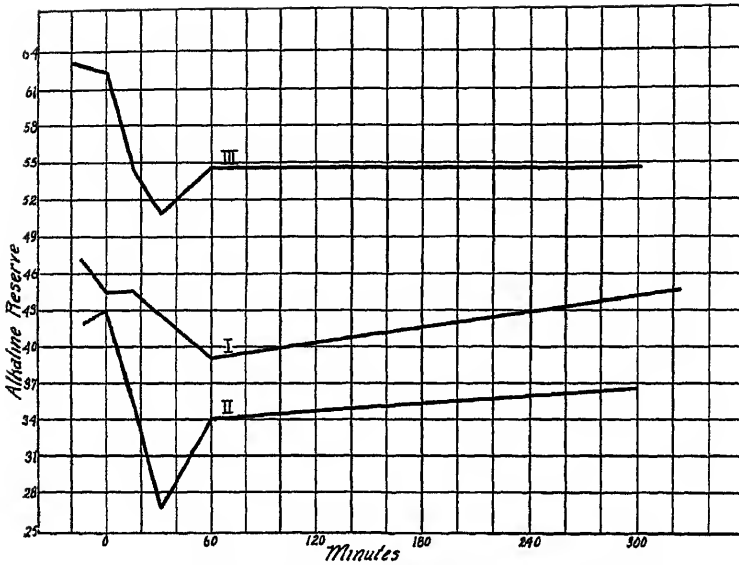
FIG 9 Curves I, II, and III (open vessel method) illustrate the effect of a first, second, and third hemorrhage on a female, not hyperimmune (Table V)
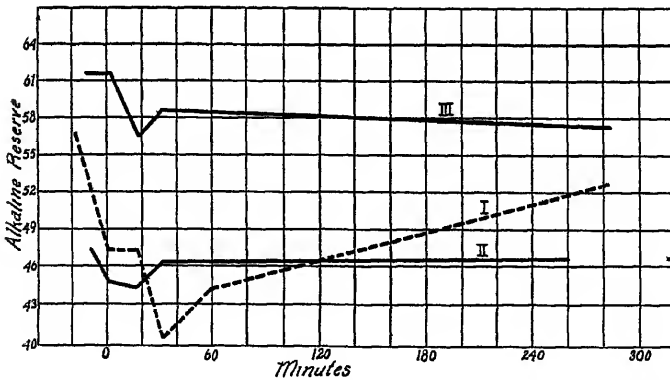
FIG 10. Curves I, II, and III (open vessel method) illustrate the effect of a first, second and third hemorrhage on a female, not hyperimmune (Table VI)

the only variation of alkaline reserve to which the animal was subject.

In many instances, the value obtained from the 5 hour sample was almost identical with that obtained from analysis of the first blood shed.   The initial value obtained at the next series of bleedings, after the interval of 5 or 7 days, might be higher or lower. These animals were kept under ordinary farm conditions, and received a mixed diet.

Influence of Hemorrhage on Other Constituents.

These striking changes in the total nitrogen content of the blood made a study of the nitrogen distribution desirable.

Taylor and Lewis,[7] working with dogs, found, when large quantities of blood were withdrawn hourly under continuous ether anesthesia, and the blood volume so lost was compensated by the injection of Ringer's solution, that the blood serum showed the following changes.   (a) A progressive decrease in the total nitrogen and total protein of the serum was less than the theoretical dilution of the blood would account for   The authors found no evidence, however, for the assumption of any synthesis of serum globulin or serum albumin during the time of the experiments.   (b) They also found such a large progressive rise in non-protein nitrogen of the serum that they concluded that the increase could not have been due to any mere washing out of the tissues.   Urea and amino nitrogen were also found to be increased with bleeding.   They said in conclusion, "It does not seem possible to resist the conclusion that the increase of non-protein nitrogen has been the result of an active process on the part of the tissues, due either to a setting free of stored amino-acids or to amino-acids derived from hydrolysis of tissue (cellular) protein or serum protein."

In the next experiment Pig 9 was bled seven times, the interval between bleedings being 5 days (with one exception). The alkaline reserve samples were collected at the usual intervals, whenever it seemed advisable to do so. Toward the end of the experiment, the blood clotted so rapidly and persistently that samples could not be obtained without cutting the tail. Non-

[7] Taylor, A E , and Lewis, H B , *J. Biol. Chem* , 1915, xxii, 71

protein nitrogen was determined by the trichloracetic acid modification of the Folin and Denis[8] method. The ammonia was distilled according to the method of Bock and Benedict,[9] and the distillate was Nesslerized. Urea was determined by Van Slyke and Cullen's[10] modification of Marshall's urease method, the ammonia being Nesslerized according to the suggestion of Rose and Coleman.[11] Chlorides were determined by the method of McLean and Van Slyke,[12] the proteins being precipitated by means of copper sulfate in alkaline solution, according to the suggestion
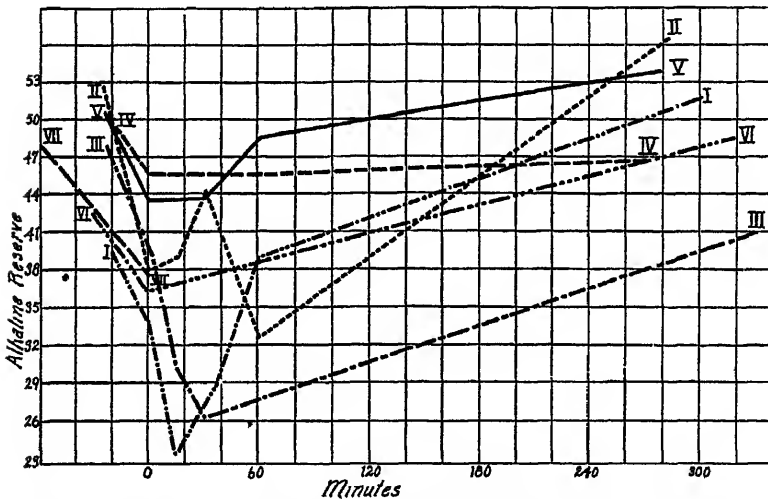
FIG 11 Curves I to VII inclusive illustrate the effect of the first to the seventh bleeding of a female whose diet was limited to corn and tap water (Table VII) All determinations were made by the open vessel method.

of Harding and Mason[13] All the determinations were run on whole blood, both on the first blood shed and also on the 5 hour samples. The results are summarized in Table VII and Fig. 11

The diet of this animal was restricted to corn and tap water,

[8] Greenwald, I , J Biol Chem , 1915, xxii, 61
[9] Bock, J C , and Benedict, S R , J Biol Chem , 1915, xx, 47
[10] Van Slyke, D D , and Cullen, G E , J Biol Chem , 1914, xix, 211
[11] Rose, A R., and Coleman, T. R , Biochem Bull , 1914, iii, 411
[12] McLean, F C., and Van Slyke, D D , J Biol Chem , 1915, xxi, 361
[13] Harding, V J , and Mason, E H , J Biol Chem , 1917, xxxi, 55

TABLE VII

Effect of Hemorrhage on Blood of Pig 9, Not Hyperimmune, Diet Limited to Corn and Water

Date	Weight lbs	Blood taken cc	Time of sampling	Alkaline reserve Volumes per cent	Maximum drop	Total nitrogen Per cent	Difference mg	Non-protein nitrogen Per 100 gm mg	Difference mg	Urea nitrogen Per 100 gm mg	Difference mg	Chlorides as NaCl Per cent	Difference	Remarks
1918 Nov 5 1st bleeding, 18 min	240	1,550	Start of bleeding	39 6		3 16		27				0 69		Uneasy toward end of bleeding, but did not struggle; fairly quiet after big bleeding, taken from crate at end of 1 hr (Fig. 11.)
			End " "	34 0										
			15 min after end	23 7	15 9									
			30 " " "	26 0										
			60 " " "	38 7										
			300 " " "	51 7		2 72	−0 44	26	−1			0 75	+0 06	
Nov 10 2nd bleeding, 27 min	258	1,000	Start of bleeding	52 7		2 57		29				0 74		Uneasy during bleeding; fairly quiet after; apparently exhausted when taken from crate at end of 1 hr
			End " "	37 8										
			15 min after end	38 7										
			30 " " "	44 0										
			60 " " "	32 4	20 3									
			285 " " "	56 4		2 35	−0 17	29	±0	9		0 76	+0 02	
Nov. 1? 3rd bleeding, 24 min	261	1,730	Start of bleeding	48 0		2 59		28				0 76		Very restless during bleeding, squealed vociferously; breathed heavily, respiration 160 per min at end of ½ hr. (normal = 25–30), typical air hunger, tail would not bleed at end of 1 hr, respiration 60, apparently normal after 5 hrs., respiration 40
			End " "	39 3										
			15 min after end	30 6										
			30 " " "	26 6	21 4									
			330 " " "	41 8		2 32	−0 27	34	+6	12	+3	0 79	0 03	

												Remarks
Nov 21 4th bleeding, 25 min	257	770	Start of bleeding.	50 4		2 54		31	13	0 73		Did not take full amount because of partial collapse of animal at previous bleeding, fairly quiet throughout, taken out from the crate between sampling
			End " "	45 8	4 6							
			30 min after end.	45 8								
			60 " " "	45 8								
			270 " " "	46 7		2 49	−0 05	32	+1 13	±0 0 69	−0 04	
Nov 27 5th bleeding, 25 min	256	1,470	Start of bleeding	50 4		2 68		24	8	0 73		Fairly quiet throughout, seemed to stand bleeding well, taken out of crate between sampling
			End " "	43 7	4 7							
			30 min after end	43 7								
			60 " " "	48 5								
			275	54 0		2 45	−0 23	28	+4 14	+6 0 72	−0 01	
Dec 2 6th bleeding, 32 min	256	1,400	Start of bleeding	42 4		2 87		29	7	0 74		Extremely difficult to get samples, because of rapid clotting, pig nervous, but did not struggle
			End " "	36 4	6 0							
			315 min after end	48 5		2 32	−0 55	32	+3 9	+2 0 71	−0 03	
Dec 7 7th bleeding, 60 min	255	1,500	Start of bleeding	47 3		2 74		27	7	0 74		Almost impossible to get samples because of rapid clotting, tail cut again to get last sample; pig apparently in good condition.
			End " "	37 3	10 0							

no salt being added to the ration. It is a recognized fact from the extensive work of Kerr, Hurwitz, and Whipple[14] that diet plays an important part in the rate of regeneration of blood. Little work has been done, however, on the effect of diet on the chemical composition of the blood. The aim of this experiment was to investigate the ability of the animal to make good the losses in alkaline reserve, due to repeated severe hemorrhages on a poor and incomplete diet which was known to be acid-producing, without the addition of salts. In this experiment no records were kept of the daily intake, and the excreta were not analyzed.

The losses in alkaline reserve were much greater the first three times the animal was bled than they were subsequently, although the data on the last bleedings are not so extensive as might be desired, due to the great difficulty in obtaining samples. When a good arterial stream could not be obtained, the samples were not collected   Apparently the alkaline reserve value of the first blood shed on any day, under the conditions of these experiments, bears no relation to such factors as the number of times the animal has been bled previously, or the amount of blood taken at previous bleedings. Whether the variations in alkaline reserve from week to week are entirely due to the experimental procedure, or are influenced by other factors to some extent is difficult to say. It happens that the initial alkaline reserve on the 1st day when the animal was bled is the lowest initial value obtained, and that the initial value on the last day is very nearly as high as any obtained. It appears, then, that this animal was capable of maintaining an efficient alkaline reserve over a period of 5 weeks on a diet which was highly acid in character, even though a total volume of blood amounting to 10 liters was withdrawn from the system during this time. So far as could be ascertained no figures obtained by recent methods are available concerning the total amount of blood in the pig. If, however, one-twelfth of the body weight is assumed to be blood, then the theoretical volume of blood in the animal at the beginning of the experiment was approximately 9 liters. Since considerably more than the probable initial blood volume was withdrawn during this experiment, it is evident that a tremendous stimulus to metabolism was afforded.

In this connection, it is of interest to note the total nitrogen content of the blood at the various stages, and the distribution

14 Kerr, W J , Hurwitz, S H , and Whipple, G H , *Am. J. Physiol.*, 1918–19, xlvii, 356, 370.

of the nitrogen, as far as data are available. Drops in the per
cent of nitrogen during the first 5 hours after bleeding are of the
same order of magnitude as those reported in the previous experi-
ment. In this case, however, there was a further drop in nitrogen
content after the first bleeding. The intervals between bleedings
were shorter in this experiment than in the preceding experiment
Beginning with the fourth bleeding, it is interesting to watch the
total nitrogen rise. The animal is evidently succeeding in restor-
ing some of the lost protein of the blood, even under these con-
ditions of repeated hemorrhage, until finally, with the seventh
bleeding, the figure falls slightly. That the protein of the blood
was actually being increased is evidenced by the data on the
non-protein nitrogen and urea nitrogen content of the blood.
Although in some cases these values were higher 5 hours after
bleeding than at the beginning of bleeding, there seemed to be
no great tendency for these forms of nitrogenous bodies to
accumulate in the blood to any significant extent. It is to be
expected that in the mobilization of nitrogenous compounds neces-
sitated by the loss of blood, unavailable portions of nitrogenous
compounds would have to be excreted; and hence that the normal
products of protein metabolism would accumulate temporarily in
the blood. Of course, the small rise in non-protein nitrogen and
urea nitrogen indicated by analysis in reality represents a very
much larger rise in these compounds when the conditions existing
in the body are remembered. The blood has been largely diluted
with tissue fluids and the total mass of actively functioning tis-
sue (the blood) has been materially reduced. In some cases, a
decrease of these bodies, as shown by analysis, might mean an
actual increase in their production. The fourth bleeding, where
a comparatively small volume of blood was withdrawn, is a case
in point. Although no salts were added to this diet which is
known to be poor in chlorides, the changes in the percentage of
chlorides were too small to be significant.

<center>DISCUSSION.</center>

There was a great variation in the reaction of different animals
to hemorrhages of the same relative magnitude, and in the
reaction of the same individual at different times. When the

same individual was bled a number of times, in all cases in which
the animal struggled the alkaline reserve values dropped lower
than in those cases in which the animal remained quiet. This
fact was so universal that it seems safe to conclude that, other
factors being equal, the drop in alkaline reserve in pigs, following
hemorrhages of this magnitude, varies directly with the amount
of struggling.   In many cases, bleeding was accomplished without
any apparent struggle.   In such cases the drop in alkaline reserve
was always small.   Doubtless, if more blood had been drawn the
drop in alkaline reserve might have been considerably greater
even though the animal remained perfectly quiet.   Since larger
hemorrhages were not attempted, no definite information can be
reported on this point.   It is true that there was considerable
variation in the amount of blood drawn at different times, but
there were so many other factors which influenced the alkaline
reserve values that differences in these values could not be attrib-
uted entirely to differences in the volume of blood drawn.   Table
VII offers a case in point.   In the case of the fourth bleeding,
when a small volume of blood was removed, there was a small
drop in alkaline reserve; but in the next bleeding, where almost
twice the volume of blood was removed, the same drop in alkaline
reserve was obtained.   In the third bleeding when an unusually
large volume was removed, there was a large drop in alkaline
reserve, but this experiment was complicated by struggling.   In
general, when comparatively large volumes of blood were removed,
there was a large drop in alkaline reserve, but in such cases the
bleeding was almost invariably associated with struggle and rest-
lessness   How much of the acidosis is attributable to actual loss
of blood and how much to the struggling is difficult to say, the
tendency to struggle doubtless being the direct result of the loss
of blood.

   The statement frequently made in the literature that hemor-
rhage is accompanied by loss in reserve alkali or by a condition
of acidosis, has been based upon experiments, as far as the author
has been able to ascertain, in which the experimental animal has
been subjected to anesthesia.   Since anesthesia is known to lower
the alkaline reserve of an animal,[5] the exact effect of the loss of
blood is not clear   In the experiments described above this
complicating factor has been eliminated.   It is true that another

factor, struggle, has been introduced.   The results obtained from
the many hemorrhages which were successfully carried out without
any apparent struggle afford evidence of the effect of loss of
blood uncomplicated by anesthesia or struggle.   The cases in
which struggle took place are of particular interest inasmuch as
more or less struggle usually accompanies the bleeding process.
Furthermore, loss of blood in general is apt to be accompanied
by struggle and nervous excitement.

Milroy's conclusion that there must have been very rapid
compensatory passage of the tissue fluid into the circulation, the
fluid first entering the circulation being extremely poor in reserve
alkali, is not confirmed by these experiments in which the pig
was the experimental animal and the Van Slyke apparatus was
the means of measuring reserve alkali.   In many cases the drop
in alkaline reserve was slight.   It is true that the hemorrhages
which Milroy described were much more severe; but it seems
reasonable to suppose that if the tissue fluids were poor in reserve
alkali there would have been greater drops in alkaline reserve
in many instances than were actually found.

The fact that one animal (Table VII and Fig. 11) when bled
seven times at intervals of 5 days was able to maintain an efficient
alkaline reserve throughout the experiment even though her diet
was restricted to corn and water affords further evidence of the
fact that acidosis is not the limiting factor which must be con-
sidered in the treatment of an animal which has lost a moderate
amount of blood.   It is true that diet undoubtedly is an extremely
important factor in the rate of regeneration of blood.[14]

The rise in urea and non-protein nitrogen in the blood after
bleeding might be caused by the breaking down of body proteins
in an effort to replace the lost circulating protein.   The urea
might function also in maintaining normal osmotic relations.

CONCLUSIONS.

1. If the blood is allowed to flow directly from an artery into
a paraffined vessel containing potassium oxalate, and if the blood
so obtained is allowed to stand a definite time ($\frac{1}{2}$ to 2 hours)

before centrifugation, the alkaline reserve values obtained by analysis of the plasma by the Van Slyke method bear a fairly definite relation to the alkaline reserve of the blood as it exists in the body. These results can be duplicated with satisfactory accuracy.

2. Alkaline reserve values obtained in this manner are lower than values obtained when the blood is brought to a definite CO_2 tension immediately after centrifugation, but, under definite conditions, are consistent with them

3 When pigs were subjected to hemorrhages amounting to approximately 1.3 per cent of body weight, a study of the alkaline reserves of these animals immediately after the hemorrhages resulted in the following observations.

(a) Hemorrhages of this magnitude were usually accompanied by a somewhat lowered alkaline reserve during the first few hours after bleeding. •

(b) When the animal remained perfectly quiet throughout the experiment, the drop in alkaline reserve was invariably small.

(c) If the animal struggled, the drop in alkaline reserve was much greater, this being noticeable soon after struggling took place.

(d) When the animal remained quiet, the maximum drop in alkaline reserve was reached within $\frac{1}{2}$ hour after the bleeding was completed. At the end of 5 hours, and often sooner, the alkaline reserve was near its original value.

(e) There was considerable variation in the reaction of different individuals.

(f) When the diet of one animal was restricted to corn and water and the animal was bled seven times at intervals of 5 days, the alkaline reserve value of the first blood shed on any day bore no relation to such factors as the number of times the animal had been bled previously, or the amount of blood (within the limits of these experiments) taken at previous bleedings

4. The total nitrogen content of the blood always fell immediately after hemorrhage. There was a distinct tendency for the urea nitrogen and the non-protein nitrogen to rise. Although one animal was bled seven times while restricted to a diet of corn and water, the percentage of chlorides in the blood remained constant.

5. On an inadequate diet (corn and water) under conditions of repeated hemorrhage there was a distinct tendency toward regeneration of blood proteins.

STUDIES .OF BLOOD REGENERATION.*

II. EFFECT OF HEMORRHAGE ON NITROGEN METABOLISM.

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INTRODUCTION

The changes in the chemical composition of the blood of the pig under conditions of repeated hemorrhage, reported in the previous paper[1] are of interest in the light of investigations of the effect of hemorrhage and acidosis on metabolism   Hawk and Gies[2] concluded that in dogs in nitrogen equilibrium hemorrhage stimulated nitrogen excretion.   Kerr and his coworkers[3] found that after plasmapharesis "The basal nitrogen metabolism shows no constant variation under these experimental conditions, with the exception of a primary rise in nitrogen elimination which occurs in the few days following the shock of the plasmapharesis." Haskins[4] found a decided rise in the amount of total nitrogen excreted on the 2 days following hemorrhage.

EXPERIMENTAL.

Pig A, a female weighing 330 pounds, was confined in a large metabolism cage, and was fed 5 pounds of corn (maize meal) each day, and 4 liters of distilled water.   (Later 5 liters of water

TABLE I

Effect of Repeated Hemorrhage on Urine of Pig A

Date	Volume	Specific gravity	Re-action	Total nitrogen	Uric acid	Uric acid N from total N	Creatinine	Creatinine N from total N	Creatine	Creatine N from total N	Ammonia N	Ammonia N from total N	Urea N	Urea N from total N	P₂O₅	P₂O₅/N
1919	*cc*		*pH*	*gm*	*mg.*	*per cent*	*gm*	*per cent*	*mg*	*per cent*	*gm*	*per cent*	*gm*	*per cent*		
Jan 11	820	1 037	5 8	19 3	309	0 48	1 56	2 17	322	0 52	2 22	11 5	13 4	69 5	4 18	0 22
" 12	1,580		5 8	16 1	311	0 58	1 64	2 74	281	0 54	3 07	19 0	11 3	70 0	4 18	0 26
" 13	2,525	1 013	6 8	16 6	282	0 51	1 41	2 29	363	0 68	2 75	16 5	11 8	70 9	2 63	0 16
" 14	2,300	1 011	6 0	14 8	308	0 62	1 41	2 55	314	0 66	2 12	14 6	10 5	70 8	3 91	0 26
" 15	2,675	1 010	6 0	17 4	366	0 63	1 69	3 00	372	0 66	2 59	14 8	12 3	70 7	4 05	0 23
" 16	2,200	1 006	6 6	10 6	243	0 69	1 13	2 88	268	0 79	1 45	-13 7	7 5	71 4	2 35	0 22
" 17	3,310	1 007	5 6	25 5	418	0 49	2 45	2 58	439	0 54	3 92	15 4	16 4	64 4	6 27	0 25
" 18	1,375	1 020	5 9	19 3	397	0 62	1 64	2 29	657	1 06	3 98	20 6	13 7	71 2	5 40	0 28
" 19	2,350	1 014	6 0	22 7	329	0 43	1 72	2 04	831	1 14	3 32	14 6	15 8	69 7	5 29	0 23
" 20	2,525	1 012	6 0	24 7	303	0 37	2 16	2 35	692	0 87	4 03	16 3	13 9	56 4	6 47	0 26
" 21	1,415	1 014	6 6	14 7	271	0 55	1 58	2 88	619	1 31	2 78	18 8	8 9	60 2	3 73	0 25
" 22	1,000	1 010	6 4	7 9			0 85	2 91	188	0 74					1 79	0 23
" 23	2,300	1 023	6 4	26 4	419	0 48	2 83	2 89	278	0 33			16 2	61 7	9 85	0 37
" 24	1,150	1 011	5 8	9 1	128	0 42	0 87	2 51	121	0 41	1 34	14 6	7 5	82 5	2 42	0 26
" 25	1,050	1 018	5 8	14 6	256	0 52	1 36	2 50	481	1 02	1 95	13 3	10 2	69 9	3 38	0 23
" 26	3,200	1 010	5 6	25 3	425	0 50	2 51	2 67	810	1 00	3 13	12 3	18 2	71 9	6 44	0 25
" 27	780	1 013	5 6	7 7	112	0 44	0 80	2 82	294	1 19	0 98	12 8	5 3	69 4	1 67	0 22
" 28	3,250	1 013	6 6	23 6	400	0 51	2 55	2 90	1,121	1 48	4 71	19 9	14 3	60 7	8 61	0 36
" 29	2,020	1 012	5 6	13 6	263	0 46	1 46	2 87	428	0 98	1 85	13 5	9 5	66 8	6 33	0 46
" 30	2,000	1 008	5 6	10 5	188	0 53	1 21	3 08	237	0 70	1 43	13 5	7 3	69 0	4 75	0 45
" 31	2,000	1 015	6 8	14 9	278	0 57	1.48	2 67	270	0 45			5 2	35 4	6 92	0 46

Date																
Feb 1	1,750	1 017	5 8	20 3	348	0 51	1 81	2 40	825	1 26	3 95	19 4	10 7	52 8	5 20	0 25
" 2	2,500	1 010	5 9	14 6	188	0 38	1 41	2 60	572	1 22	2 00	13 7	10 5	71 8	4 26	0 29
" 3	2,500	1 017	5 6	20 0	441	0 46	2 58	2 40	1,513	1 63	7 13	24 6	16 1	55 5	10 83	0 37
" 4	2,700	1 016	5 6	21 7	377	0 52	1 97	2 44	923	1 32	2 82	13 0	15 4	70 9	7 16	0 33
" 5	1,950	1 010	5 6	11 7	203	0 52	1 05	2 41	435	1 15	1 49	12 7	8 7	74 1	4 71	0 40
" 6	2,120	1 017	5 6	19 7	356	0 54	1 82	2 48	542	0 85	5 50	27 9	10 5	53 2	7 06	0 36
" 7	3,500	1 012	5 6	29 3	464	0 47	2 77	2 54	956	1 01	6 87	23 4	17 1	58 3	10 29	0 35
" 8	1,475	1 020	5 8	20 1	455	0 68	1 58	2 19	1,053	1 63	1 79	8 9	14 4	71 5	3 92	0 19
" 9	2,550	1 018	6 1	10 6	177	0 50	1 27	3 22	537	1 57	1 94	18 2	12 3		3 46	0 33
" 10	3,000		6 4	29 3	482	0 49	2 50	2 29	1,018	1 08	5 22	17 7	19 0	65 0	7 52	0 26
" 11	2,450	1 009	6 0	12 7	223	0 52	1 13	2 39	605	1 47					3 71	0 29
" 12	1,000	1 013	5 6	18 5	330	0 53	2 20	3 19	946	1 59	2 84	15 6	12 3	66 3	5 78	0 31

65

were given.) No salts were added. After the animal had been kept under these conditions for 5 days, daily collections of the complete urinary excretion were made, and the following determinations were made volume, specific gravity, hydrogen ion concentration by the Clark and Lubs[5] method, total nitrogen by the Kjeldahl method, uric acid by the Benedict-Hitchcock[6] modification of the Folin-Denis method, creatinine by Folin's colorimetric method, creatine by the Folin-Benedict[7] method, ammonia nitrogen by Folin's aeration method, urea by Van Slyke and Cullen's[8] modification of Marshall's urease method, the ammonia formed being titrated, and total phosphates by titration with uranium acetate The animal was bled four times at intervals

TABLE II

Period Averages of Urinary Analysis of Pig A

Date.	Total nitrogen	Creatinine	Creatinine N from total N.	Creatine as creatinine	Creatine N from total N.	Uric acid.	Uric acid N from total N	Ammonia N.	Ammonia N from total N.	Urea N	Urea N from total N	Phosphates as P₂O₅	P₂O₅/N
1919	*gm*	*gm*	*per cent*	*mg*	*per cent*	*mg*	*per cent*	*gm*	*per cent*	*gm*	*per cent*	*gm.*	
Jan 11–17	17 2	1 61	2 52	337	0 61	320	0 56	2 59	15 4	11 9	69 2	3 95	0 23
" 18–24	17 8	1 66	2 51	484	0 84	358	0 60	3 09	17 0	12 0	68 0	4 99	0 28
" 25–31	15 8	1 62	2 77	520	1 03	274	0 52	3 06	19˙4	10 1	64 1	5 45	0 34
Feb. 1– 7	20 9	1 90	2 44	823	1 23	340	0 49	4 25	20 3	12 7	60 8	7 07	0 34
" 8–12	18 3	1 73	2 64	832	1 47	334	0 54	2.95	15 2	15 2	67 6	4 88	0 28

of 7 days, in the ordinary manner from the tail, approximately 6 cc. of blood per pound of body weight being taken. The results of the daily analysis of the 24 hour collection of urine are given in Table I, and the period averages of these same data in Table II. The breaks in Table I indicate the occurrence of hemorrhage. The results of the blood analysis are given in Table III. The determinations, in this case, were made as described previously,[1] with the exception that the ammonia in the urea determinations was titrated

[5] Clark, W. M., and Lubs, H A , *J Bacteriol* , 1917, ii, 1

[6] Benedict, S R , and Hitchcock, E H , *J Biol Chem* , 1915, xx, 619

[7] Benedict, S R , *J Biol Chem* , 1914, xviii, 191

[8] Van Slyke, D. D , and Cullen, G E , *J Biol Chem* , 1914, xix, 211.

instead of Nesslerized, a 5 cc. sample of blood being taken. The alkaline reserve values from this series of bleedings are plotted in Fig. 1. In general, the data illustrate the points which have already been discussed.[1] Of particular interest are the results

TABLE III.

Effect of Hemorrhage on Blood of Pig A

Date	Blood taken	Duration of bleeding	Time of sampling	Alkaline reserve	Total N	Urea N per 100 cc of blood	Remarks
1919	cc	min		vol. per cent	per cent	mg	
Jan 17	1,900	16	Beginning of bleeding	48 1	2 89	26 3	Fairly quiet throughout.
			End of bleeding	44 9			
			120 min after end	44 9	2 59	32 0	
" 24	2,100	18	Beginning of bleeding.	50 7	2 45	32 4	Fairly quiet throughout
			End of bleeding.	50 7			
			15 min after end	50 7			
			120 min. " "	50 7	2 20	35 6	
" 31	1,850	30	Beginning of bleeding	51 6	2 55	33 7	Quiet at first. Struggled considerably toward end of bleeding. Had to cut the tail to get the 15 min sample
			15 min after end.	37 4			
			120 " " "	51 4	2 16	38 5	
Feb 7	1,850	19	Beginning of bleeding	53 4	2 53	26 0	Quiet at first Struggled toward end of bleeding Left pig in crate to take the hour sample but could not get a good stream Blood clotted very rapidly. Animal quite exhausted but did not develop air hunger
			15 min after end	20 6	2 11	26 5	

obtained at the second bleeding, January 24.   In this case, there
was no drop in reserve alkali at all, even though a larger amount
of blood was taken than at any other bleeding in the series.   In
the case of the fourth bleeding, February 7, the drop was extremely
rapid, but there were no symptoms of air hunger, and the animal
apparently suffered no untoward effects from the experiment.

The total nitrogen content was influenced by the hemorrhages
much as in the previous experiments.   Urea nitrogen was dis-
tinctly higher at the end of 2 hours than at the beginning of bleed-
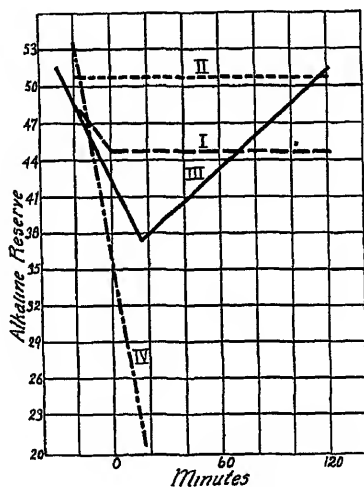
Fig 1   Curves I, II, III, and IV illustrate the effect of the first, second,
third, and fourth hemorrhage on the alkaline reserve of Pig A, which was
kept in a metabolism cage and restricted to a diet of corn and distilled
water (Tables I, II, and III)   All determinations were made by the open
vessel method

ing.   In the last bleeding (February 7) a determination of urea
nitrogen was made at the end of 15 minutes.   At this time the
urea nitrogen was the same (a very slight rise) as at the beginning
of the bleeding, although the total nitrogen had dropped con-
siderably.   In general, bleeding had the effect of raising the urea
content of the blood within a short time after the blood was shed,
and there was a tendency for the urea to remain high, although
it dropped back to the original figure before the end of the ex-
periment, in spite of the fact that the hemorrhages were regularly

repeated at intervals of 7 days. During this time of high urea content in the blood there was no increase in the excretion of urea in the urine (Table II).

In order to study the effect of hemorrhage on endogenous nitrogen metabolism, another animal, Pig B, a female, weighing 360 pounds, was confined in a metabolism cage, and after being fasted for 3 days, was offered distilled water and starch to which sufficient sodium chloride had been added to make the ration palatable enough to induce the animal to eat   After 3 days of starch feeding the sodium chloride was discontinued and daily collections of the urine were made.   During the remainder of the experiment the animal received 4 pounds of corn-starch daily and about 5 liters of distilled water.   In this way, exogenous nitrogen metabolism was reduced to a minimum, but sufficient calories were provided to cover the energy expenditure of the animal.   The results of the daily analysis of the complete urinary excretion are given in Table IV and the period averages of these results in Table V.   This animal was bled twice, and 6 cc. of blood per pound of body weight were taken, the interval between bleedings being 5 days.   The alkaline reserve values are given in Table VI.

Soon after the pig was taken from the crate at the end of the 1 hour period, she developed a severe case of air hunger.   The respiration was shallow and rapid, about 180 a minute.   She refused food and water and was extremely nervous.   After several hours an attempt was made to give the animal distilled water by rectum.   Several hundred cc. were retained.   There was no urine during the first 24 hours after the bleeding   The urine on the following day was collected and analyzed in two separate portions.   It was very deeply pigmented.   On the following day, March 3, the pig was apparently much better.   The respiration seemed normal   She had no difficulty in moving about, but continued to refuse food and water: Consequently the experiment was discontinued, and in an effort to save the animal 3 liters of milk in which 10 gm of sodium acetate had been dissolved were administered by stomach tube.   At night the pig was apparently normal, but was found dead in the morning, March 4, death being caused by a severe hemorrhage of the lungs   There was no reason to believe that death was due directly to the specific effects of the experimental procedure

TABLE IV.

Daily Analysis of Urine of Pig B

Date	Urine	Reaction	Specific gravity	Total N	Creatinine	Creatinine N from total N	Creatine as creatinine	Creatine N from total N	Uric acid	Uric acid N from total N	Phosphates as P_2O_5	$\frac{P_2O_5}{N}$
1919	cc	pH		gm	gm	per cent	mg	per cent	mg.	per cent	.mg	
Feb 20	0											
" 21	5,200	6 2	1 008	12 9	3 93	8 19	465	1 12	532	1 23	3 44	0 266
" 22	4,150	6 6	1 006	9 5	1 90	5 46	432	1 41	374	1 17	2 63	0 275
" 23	3,300	6 6	1 005	7 5	2 20	7 88	392	1 63	326	1 30	1 66	0 222
" 24	2,630	6 6		4 6	1 47	8 60	112	0 75	189	1 23	0 92	0 201
" 25	2,000	6 0	1 009	9 0	2 48	7 38	392	1 35	422	1 40	2 12	0 235
" 26	2,900	6 4	1 006	7 6	1 63	5 70	347	1 41	249	0 97	0 95	0 124
" 27	2,725	6 0	1 006	7 3	1 74	6 35	204	0 86	252	1 02	1 80	0 244
" 28	4,300	6 0	1 003	7 8	2 53	8 66	358	1 42	288	1 10	1 83	0 233
Mar 1	3,700	5 8	1 002	5 0	2 22	11 83	345	2 12	304	1 81	1 13	0 225
" 2	0											
" 3	2,960			27 0	2 93		7,202	8 31	580	0 64	6 60	0 244
" 3a*	1,460	6 0	1 021	12 2	1 58	3 47	3,789	9 62	292	0 71	3.50	0 28
" 3b*	1,500	6 2	1 018	14 7	1 34	2 46	3,412	7 22	288	0 59	3 09	0 21

* The urine excreted during this day was collected and analyzed in two separate portions, *a* and *b;* *a* represents the period from 8 a m , March 2, to 5 p m , March 2, *b* represents the period from 5 p m , March 2, to 8 a m. March 3.

The breaks in the table indicate the occurrence of hemorrhage.

TABLE V

Period Averages of Urinary Analysis of Pig B

Date	Total N	Creatinine	Creatinine N from total N	Creatine as creatinine	Creatine N from total N	Uric acid	Uric acid N from total N	Phosphates as P_2O_5	$\frac{P_2O_5}{N}$
1919	gm	gm	per cent	mg	per cent	mg	per cent	gm	
Feb 20–24	6 9	1 904	7 39	280	1 26	284	1 23	1 73	0 25
" 25–Mar 1	7 4	2 125	7 70	329	1 33	303	1 18	1 57	0 20
Mar 2–3	13 5	1 467	2 92	3,600	8 31	290	0 64	3 30	0 24

TABLE VI.

Effect of Hemorrhage on Blood of Pig B.

Date	Blood taken	Duration of bleeding	Time of sampling	Alkaline reserve	Total N	Remarks
1919	*cc*	*mın*		*vol per cent*	*per cent*	
Feb. 24	2,100	30	Beginning of bleed-ing	53 8	3 06	Pig was quiet until the last ½ hr. Then she struggled intermittently Seemed exhausted at the end of the hour. Respiration was heavy and deep, about 70 per min.
			End of bleeding.	56 0		
			15 mın after end.	56 0		
			30 " " "	53 8		
			60 " " "	43 9	2 86	
Mar. 1	2,100	45	Beginning of bleed-ing	49 0	2 74	Pig fairly quiet while in the crate. Struggled a little intermittently during the last ½ hr
			15 mın after end	36 6		
			30 " " "	44 8		
			60 " " "	30 3	2 45	

DISCUSSION.

The data obtained on the effect of repeated hemorrhage on the composition of the blood of pigs (Tables III and VI, and Figs. 1 and 2) supplement the data already reported in the previous paper. In one case, Fig. 1, Curve II, there was no drop in alkaline reserve after hemorrhage.

The data obtained from the urinary analysis of Pig A (Tables I and II) give no definite indication of increased nitrogen excretion due to hemorrhage. Although the collections were made at the same time each day, it is evident that in some cases the urine so obtained in reality represented a longer period than 24 hours, and in other cases a shorter period. When period averages are considered, the values vary in each direction and therefore cannot be interpreted as demonstrating a significant change. In the case of Pig B, however, where the nitrogen excretion represented endogenous metabolism only, there is a definite indication of increased nitrogen metabolism. This fact might be interpreted

as meaning that when no protein was furnished from which the animal could obtain fragments necessary to replace the lost tissue, there was greater autolysis of body tissue to made good the loss and also greater activity of the blood-making organs with a correspondingly increased excretion of the end-products of the metabolism of these organs.   Since the data on the effect of hemorrhage on endogenous nitrogen metabolism is so limited, and since the experiment terminated fatally to the animal, leaving room for doubt as to the exact nature of the cause of death, too much stress should not be placed on the results
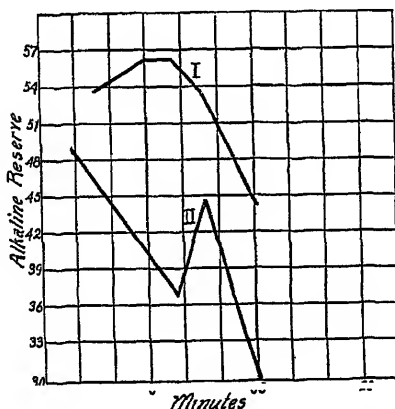
FIG 2. Curves I and II (open vessel method) illustrate the effect of the first and second hemorrhages on Pig B   The pig was kept in a metabolism cage and the diet was restricted to starch and water   The last point on Curve II represents a sample of blood taken just before severe symptoms of air hunger set in (Tables IV, V, and VI)

In the case of Pig A the effect of hemorrhage on uric acid metabolism was too small to be significant.   According to the theory of Lewis and his coworkers,[9] amino-acids stimulate all cellular metabolism, with the result that after protein ingestion (in man) there is an increased excretion of uric-acid.   If hemorrhage causes autolysis of tissue protein, an increased excretion of uric acid might be expected.   Uric acid, however, is not the only end-product of purine metabolism in the pig.   Under

[9] Lewis, H B , Dunn, M.S , and Doisy, E A , J Biol Chem , 1918, xxxvi, 9

the stress produced by hemorrhage, it is conceivable that purine metabolism might be carried out of its normal course   In the case of Pig B, where uric acid excretion was entirely endogenous, the effect of the first bleeding is not clear.   If amino-acids stimulate uric acid excretion, it would be expected that total nitrogen and uric acid would run parallel and consequently that the per cent of uric acid nitrogen would not rise.   The fact that there was an increased uric acid excretion on the day following bleeding does not necessarily mean that there was an increased production of uric acid during that day, because of the uncertainty of the
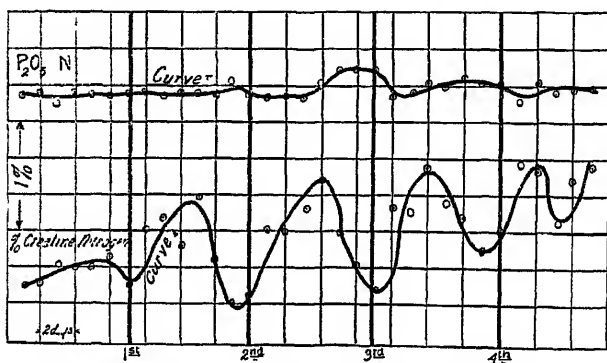
FIG. 3   Curve I represents the daily urinary fluctuation in the per cent of creatine nitrogen of total nitrogen   Days on which bleeding took place are represented by vertical lines   Bleeding stimulates creatine elimination Curve II represents the daily fluctuation in the ratio of P_2O_5 to nitrogen This ratio rises after the 18th day, and falls again on the day following bleeding (Pig A, Table I)

accuracy with which the samples represented a 24 hour period. The period averages show a slight increase in uric acid production after the first bleeding, too small, however, to be significant. If the first collections of urine after the second bleeding (there was no urine on the first day) represented a 48 hour excretion, then the actual amounts of uric acid excreted remained remarkably constant.   In any case, the uric acid excretion was not at all proportional to the total nitrogen.   Although there is no experimental evidence for this view, it is conceivable that those tissues which are comparatively low in nucleic acid, such as the

muscles, were autolyzed to a great extent, with the result that uric acid excretion was not proportional to nitrogen excretion.

Much emphasis has been laid on the constancy of the creatinine excretion for each individual. Haskins,[4] however, found a significant decrease in the creatinine excretion following hemorrhage. It is true that in the case of Pig A there was considerable daily variation in the amounts of creatinine excreted. The percentages of creatinine nitrogen were fairly constant, however, particularly when period averages are considered. It happens that in each case there was a slightly diminished excretion of creatinine nitrogen, expressed as percentage of total nitrogen, on the day following bleeding. These drops fall well within the limits of daily variation, however, and therefore cannot be considered significant. An apparent fall in the per cent of creatinine nitrogen might, of course, be due to a corresponding increase in total nitrogen excretion. This experiment, therefore, affords no evidence that repeated hemorrhages of this magnitude influence the excretion of creatinine. In the case of Pig B, the period averages might seem to indicate that there was a significant reduction in creatinine output after the second bleeding. This effect, also, is directly dependent upon the accuracy with which this collection of urine represented the excretion of the 48 hours following bleeding. Since this point is in doubt, there is little reason to think that the creatinine value was actually lowered by bleeding.

In the case of Pig A, there seems to be no doubt that hemorrhage increased the excretion of creatine when both actual amounts of creatine and percentage of creatine nitrogen are considered. According to the conception of Underhill,[10] that acidosis is a determining factor in the excretion of creatine (in rabbits) this result might be explained on the basis of the fact that hemorrhage is accompanied by lowered alkaline reserve. Several facts, however, are in opposition to this view. The data here presented on the effect of hemorrhage on alkaline reserve, ammonia excretion, and the hydrogen ion concentration of the urine, all point toward the fact that the acidosis produced by this experimental procedure was usually slight and of brief duration. Steenbock and Gross,[11]

[10] Underhill, F P , J Biol Chem , 1916, xxvii, 127

[11] Steenbock, H , and Gross, E G , J Biol Chem , 1918, xxxvi, 265

working with pigs, found that intensive protein-feeding resulted
in increased creatine production even during alkalosis. The con-
clusion seems more plausible, therefore, that the increased creatin-
uria was caused by an alteration in the course of nitrogen metab-
olism due to hemorrhage.

When the exogenous nitrogen metabolism was eliminated (Pig
B), the second bleeding brought about a tremendous rise in the
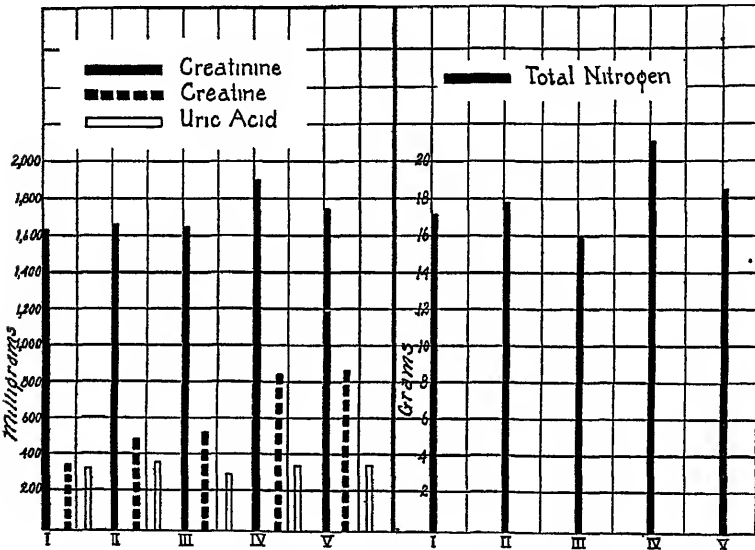
Fig. 4 The vertical lines represent the averages of the actual amounts
of creatinine, creatine, uric acid, and total nitrogen respectively excreted
during the various periods of the experiment. Line I represents the fore
period, Line II the period between the first and second hemorrhages, Line
III the period between the second and third hemorrhages, etc (Pig A,
Table II).

amount of creatine excreted, and also in the per cent of creatine
nitrogen. It is true that other unknown factors beside simple
hemorrhage may have entered into this effect. The picture sug-
gests itself, however, that when the blood stream is suddenly
flooded with the fragments resulting from the breaking down of
protein of either endogenous or exogenous origin in such large
amounts that the organism is unable to metabolize them in the

ordinary way, an increased creatine production and excretion results

There are considerable data in the literature to show that acid-feeding, if sufficiently intensive, produces an increased excretion of phosphates in the urine in animals   Goto[12] found that acid-feeding produced no definite influence on the distribution of phosphates between urine and feces of rabbits.   The data for Pig A show that the phosphate excretion in the urine was fairly constant until the 18th day of the experiment.   At this point the ratio $\dfrac{P_2O_5}{N}$ rose definitely.   This fact shows clearly, if it is assumed that there was no corresponding fall in fecal phosphate, that alkaline phosphates were being lost from the body, presumably to help neutralize the excess acid formed.   The fact that the next two bleedings were followed on the next day by a decreased $\dfrac{P_2O_5}{N}$ ratio may indicate that the readily available phosphates were exhausted at the time, due to loss of large quantities of phosphoric acid in the blood withdrawn   In the case of Pig B, the $\dfrac{P_2O_5}{N}$ ratio remained fairly constant throughout   This effect would be expected on account of the short duration of the experiment: but it is of particular interest inasmuch as the nitrogen figure rose so high after the second bleeding, and no phosphates were supplied with the food.

It is a rather remarkable fact that the hydrogen ion concentration of the urine of Pig B suffered so little change after the second bleeding during the 48 hours when the animal was suffering from acute air hunger.   .

In the alkaline reserve values (Table VI) obtained after the second hemorrhage is found an interesting example of a preliminary fall in alkaline reserve with the usual attempt at rapid restoration, followed by a second sharp fall   It is unfortunate that samples of blood could not be drawn during the entire period of air hunger.

[12] Goto, K , *J. Biol Chem* , 1918, xxxvi, 355

CONCLUSIONS

1 On a diet of corn and water, under conditions of repeated hemorrhage, the creatine excretion of two animals (pigs) was definitely increased.   This effect was cumulative.

2. When the diet was restricted to starch and water, the second hemorrhage caused an increased excretion of total nitrogen, phosphates, and creatine.

3. The theory that hemorrhages amounting to 6 cc per pound of body weight are not necessarily accompanied by a severe grade of acidosis is supported by the following observations

(a) The drops in alkaline reserve were slight and of short duration

(b) There was no great increase in the ammonia nitrogen excretion after hemorrhage.

(c) The hydrogen ion concentration of the urine was not definitely influenced by hemorrhage.

ANIMAL CALORIMETRY.

SIXTEENTH PAPER

THE INFLUENCE OF LACTIC ACID UPON METABOLISM.*

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The experiments of this laboratory have demonstrated that a causative element of the great increase in heat production after meat ingestion is due to the stimulus provided by the acid products of protein metabolism. Thus, when glycocoll and alanine are given to a dog the metabolism increases greatly (1, 2). The increased metabolism is not due to the process of deamination or of urea formation because the administration of glutamic acid (1) or of asparagine (3) (the latter having the same NH_2 content as glycocoll) is without influence upon metabolism. The increased function of the kidney is without influence, for neither urea nor sodium chloride, when administered in aqueous solution, has any power to increase the basal metabolism (4). These experiments also exclude an increased osmotic exchange between the blood stream and the cells as a cause of the specific dynamic action.

It might be objected that glycocoll and alanine, which sometimes produce vomiting, cause an increased metabolism through gastric or intestinal irritation, but urea and salt solutions also sometimes produce vomiting; yet when they are retained by the stomach there is no rise in heat production after their administration. Benedict and Emmes (5) have shown that the administration

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* An abstract of this work was published as, Calorimétrie comparée de l'ingestion de viande, d'acide lactique et d'alanine chez l'animal (Lusk, G., *Compt. rend. Acad.*, 1919, clxviii, 1012)

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tration of severe cathartics does not cause the heat production to increase   Furthermore, the increased metabolism is not due to the sudden availability of glycocoll or of alanine as providers of heat because a considerable increase in metabolism is achieved when these substances are administered in phlorhizin glycosuria, under which conditions they are completely converted into glucose and urea without the liberation of energy by oxidation (2).   The amino-acids themselves are not stimuli to metabolism, for Rubner (6, *a*) has correctly shown that that quota of the ingested protein which is·absorbed as amino-acids and deposited as new protein in the body exercises no *specific dynamic* action, in other words, does not raise the heat production

There is a fundamental distinction between the heat production induced by protein and that brought about by carbohydrates. Thus, when a fasting dog is caused to perform a measured quantity of work, his heat production is the same as when he is given sugar in abundant supply.   If the dog is given meat and caused to do the same amount of work as before his energy production will be raised above the basal level by the increment of the *specific dynamic* influence of the meat superimposed upon the increment of metabolism produced by the performance of the work (7).

In this fact lies the proof that the character of the cause of the increased heat production after giving meat and carbohydrate is essentially different.

That the primary metabolites are different is also indicated by the fact that, though fructose, when given in phlorhizin glycosuria, is converted into glucose and as such completely eliminated in the urine, the intermediary metabolites involved in this chemical transformation do not cause any increase in the metabolism of the dog (2).

In this connection it is of interest to note that the alkaline reserve of the blood falls after the administration of meat, which indicates the production of acid metabolites; this is not in evidence after the ingestion of a vegetarian diet (8).   Atkinson and Lusk (3) have demonstrated that 200 cc. of 0.4 per cent of hydrochloric acid will increase the heat production in the dog from 20 to 21.25 calories per hour.

Furthermore, in phosphorus poisoning, a condition in which large quantities of lactic acid are produced, the heat production

is increased (9).   It also appears probable that the rise in heat production in severe anemias is due to the pathological production of lactic acid from carbohydrates, which production may be assumed to take place only in the absence of an adequate oxygen supply to the cells (10).

These considerations naturally lead one to the conclusion that lactic acid itself, if given to a dog, might cause an increase in the heat production, just as ingested alanine does, a presumption which proved to be justified,

Methods.

The dog was given the "standard diet" at 5 p.m.; then either (1) the "basal metabolism" was determined by the calorimeter on the following morning, 18 hours or more after food ingestion, or (2) the material to be investigated was given by stomach tube during the morning and the dog was then placed in the calorimeter at an environmental temperature of 25 or 26°C.   The results in the two instances could be compared (2).

EXPERIMENTAL.

It was found that when 10 cc. of d-l-lactic acid containing 8 gm of lactic acid in solution were dissolved in 500 cc. of water, the dog invariably violently vomited the material.   In one instance when 500 cc. of pure warm water were administered the dog regurgitated a considerable amount of the ingesta as though the mass were too great to be retained   However, when 500 cc. of water containing 2.5 gm. of Liebig's extract of meat were given the fluid was always retained.   Whether this difference was due to the saline content or to the flavoring extractives in the Liebig's extract is unknown, but it is certain that beef broth is more readily retained in the stomach than plain water.   When 2 5 gm of Liebig's extract were added to the 1 6 per cent solution of lactic acid described above the fluid was retained in about half the instances. At one time, after quite frequently repeated administrations, the lactic acid solution was persistently vomited whenever given and the completion of the work was delayed until the dog's stomach had recovered from its abnormal irritability.   When the lactic acid

was diluted with 400 cc. (instead of 500 cc.) of water the resulting
2 per cent solution was vomited.   The solution was always given
at a tempeiature of 38°C.

Series 1.

In this short seiies of expeiiments a comparison of the metab-
olism, after giving 8 gm. of lactic acid, with that found after
giving 8 gm. of alanine was made as shown in Table I.

TABLE I

Dog XVIII, Series 1

No. of experiment	Date	State of nutrition	Hours of experiment	R Q	Calories per hour		Increase over indirect basal (17 8 calories)
					Indirect	Direct	
	1918						
20	Dec 26	Basal metabolism	1	0 84	17 8	15 9	
21	" 27	Lactic acid, 8 gm , Lie-big's extract, 2 5 gm., water, 500 cc	2	0 93	20 9	21 0	+3 1
22	" 31	Alanine, 8 gm , Liebig's extract, 2 5 gm , water, 150 cc	2	0 90	19 3	19 2	+1 5

The urinary nitrogen in Experiment 20 was 0.085 gm. per hour;
in Experiment 25 it was 0.113 gm. per houi.   In Experiment 22
it was assumed that the metabolism of body protein furnished the
same 0.113 gm. of nitrogen per hour as in Experiment 25, that 70
per cent of the total amount of *d-l*-alanine was metabolized, and
that of this 23 per cent was metabolized in the 2nd, and 21 per
cent in the 3rd hour after the ingestion of the material (11).

Since lactic acid and glucose yield almost the same number of
calories per gm. and both yield a respiiatory quotient of 1.00, the
calculation of the heat production by the indirect method after
the usual manner is entirely justified.

The experiments (Table I) indicate that 8 gm. of lactic acid
administered in 500 cc of water increase the metabolism from
17 8 to 20.9 calories, an inciement of 3 1 calories, whereas 8 gm.
of alanine given in 150 cc. of water cause an increase of only 1.5

calories. Unfortunately, no more alanine was available The discrepancy between the results was due to the difference in the quantity of water ingested, as appears in the second series of experiments.

Series 2.

It was stated by Rubner (6, *b*) and substantiated in this laboratory that the ingestion of a solution of Liebig's extract of meat

TABLE II

Dog XVIII, Series 2

No. of experiment	Date	State of nutrition	Hours of experiment	R Q	Calories per hour		Increase over indirect (174 calories).
					Indirect	Direct	
	1919						
34	Feb 24	Meat, 1,080 gm	2	0 80	34 0	34 1	16 6
37	" 28	Basal metabolism	2	0 85	17 3	17 0	
39	Mar 3	" "	3	0 85	17 6	17 2	
40	" 4	Lactic acid, 8 gm , Liebig's extract, 2 5 gm , water, 500 cc	3	0 88	19 4	18 3	2.0
41	" 5	Basal metabolism	3	0 82	17 5	16 7	
42	" 11	Liebig's extract, 2 5 gm ; water, 500 cc	3	0 80	18 5	17 6	1.1
43	" 12	Basal metabolism	2	0 81	17 1	17 0	
44	" 13	Liebig's extract, 2 5 gm , water, 500 cc	3	0 78	18 2	17 5	0 8
45	" 14	Liebig's extract, 2 5 gm , water, 150 cc	3	0 84	17 0	16 9	
	-	Average .			19 6	19 1	

was without influence upon the heat production.   The increase in metabolism after giving meat extract to Dog XVIII, as shown in Table II, seemed at first difficult of interpretation.   However, it was demonstrated by Experiment 45 that when the volume of the fluid administered was reduced from 500 cc. to 150 cc. the trustworthiness of the older experiments was confirmed, for there was then no rise in the heat production.

However, whenever the larger volume of fluid was given there was a rapid elimination of it by the kidney, as is shown in Table III.

TABLE V —*General*

Date	Experiment No	Time	CO_2	O_2	R Q	H_2O	Urine, basal N	Calories					Percentage of heat loss by evaporation of water
								Protein	Alanine	Non-protein	Indirect	Direct	
			gm	*gm*		*gm*	*gm*						
1918													
Dec 26	20	11 26–12 26	6 10	5 29	0 84	7 04	0 085	2 25		15 52	17 77	15 93	25
" 27	21	11 30–12 30	8 24	6 07	0 99	7 93	(0 113)*	3 00		18 09	21 09	21 37	21
		12 30– 1 30	7 35	6 13	0 87	7 30	(0 113)	3 00		17 77	20 77	20 68	20
" 31	22	11 30–12 30	7 40	5 69	0 95	6 82	(0 113)	3 00	4 47	11 84	19'31	20 25	20
		12 30– 1 30	6 80	5 78	0 85	6 82	(0 113)	3 00	4 08	12 21	19 29	18 11	22
1919													
Feb 24	34	12 45– 2 45	12 59	7 71	0 83	18 81	1 57	41 62		−7 40	34 22	35 72	53
		1 45– 2 45	11 64	7 71	0 77	18 45	1 57	41 62		−7 95	33 67	33 51	55
" 28	37	11 00–12 00	6 30	5 52	0 83		0 169	4 48		13 88	18 36	17 52	
		12 00– 1 00	5 47	4 85	0 86	6 50	0 169	4 48		11 75	16 23	16 37	23
Mar 3	39	11 00–12 00	6 39	5 48	0 85	6 88	0 154	4 08		14 27	18 35	17 34	2
		12 00– 1 00	5 97	5 09	0 85	6 13	0 154	4 08		12 96	17 04	15 64	23
		1 00– 2 00	6 05	5 19	0 85	6 28	0 154	4 08		13 28	17 36	18 67	20
" 4	40	11 00–12 00	6 96	5 64	0 90		(0 154)	4 08		15 04	19 12	17 43	
		12 00– 1 00	7 26	5 81	0 91	7 05	(0 154)	4 08		15 74	19 78	17 92	21
		1 00– 2 00	6 75	5 81	0 85	6 46	(0 154)	4 08		15 38	19 46	19 50	20
" 5	41	11 00–12 00	5 89	4 95	0 86		0 145	3 84		12 78	16 62	16 67	
		12 00– 1 00	6 07	5 47	0 81	7 28	0 145	3 84		14 28	18 12	16 46	26
		1 00– 2 00	5 94	5 39	0 80	6 83	0 145	3 84		13 98	17 82	16 99	24
" 11	42	11 00–12 00	6 19	5 52	0 82		(0 145)	3 84		14 49	18 33	16 57	
		12 00– 1 00	5 81	5 46	0 77	6 77	(0 145)	3 84		14 09	17 93	17 32	23
		1 00– 2 00	6 37	5 81	0 80	6 65	(0 145)	3 84		15 38	19 22	18 90	21
" 12	43	11 00–12 00	5 78	5 06	0 83	6 14	(0 145)	3 84		13 00	16 84	17 14	21
		12 00– 1 00	5 77	5 24	0 80	6 16	(0 145)	3 84		13 48	17 32	16 83	22
" 13	44	11 00–12 00	6 11	5 26	0 84			3 84		13 74	17 58	18 43	
		12 00– 1 00	5 83	5 66	0 75	6 60	(0 145)	3 84		14 64	18 48	17 12	21
		1 00– 2 00	5 96	5 71	0 76	6 61	(0 145)	3 84		14 85	18 69	16 98	22

Nitrogen values in parentheses are assumed from nearby records of the basal values."

Summary of Dog XVIII

Morning weight.	Behavior of dog	Food
kg 11 35	Quiet	Basal metabolism.
11 31	Slight movement Quiet	8 gm lactic acid; 2 5 gm meat extract; 500 cc. water at 10 35 a m
11 26	Slight movement between the 2 hours	8 gm. alanine, 2 5 gm meat extract; 150 cc. water at 10 30 a m
12 07	Several movements " "	1,080 gm meat at 9 a m
11 95	Slight movement Quiet	Basal metabolism.
11 77	Slight movement Quiet Slight movement	Basal metabolism
11 77	Quiet. " "	8 gm. lactic acid, 2 5 gm meat extract, 500 cc water at 10 17 a m
	" Slight movement " "	Basal metabolism
11 55	Quiet " Slight movement	2 5 gm meat extract; 500 cc. water at 10 20 a m.
11 50	Quiet "	Basal metabolism
11 53	" " "	2 5 gm meat extract, 500 cc water at 10 15 a m.

TABLE V—

Date	Experiment No	Time	CO₂	O₂	R Q	H₂O	Urine, basal N	Calories					Percentage of heat loss by evaporation of water
								Protein	Alanine	Non-protein	Indirect	Direct	
1919			*gm*	*gm*		*gm*	*gm*						
Mar 14	45	11 30–12 30	5 82	4 83	0 88	5 65	(0 145)	3 84		12 41	16 25	16 37	20
		12 30– 1 30	5 98	5 40	0 81	5 84	(0 145)	3 84		14 04	17 88	16 28	21
		1 30– 2 30	5 93	5 05	0 85	5 34	(0 145)	3 84		13 07	16 91	18 12	17
May 9	57	11 45–12 45	5 81	4 90	0 86		0 150	3 98		12 46	16 44	15 39	
		12 45– 1 45	5 90	4 57	0 94	7 54	0 150	3 98		11 64	15 62	17 00	27
		1 45– 2 45	6 01	5 35	0 82	7 44	0 150	3 98		13 79	17 77	17 98	24
" 10	58	11 00–12 00	5 70	4 58	0 91		0 155	4 11		11 40	15 51	13 76	
		12 00– 1 00	5 95	4 71	0 92	6 92	0 155	4 11		11 90	16 01	16 33	25

Concluded

Morning weight	Behavior of dog	Food
kg 11 55	Quiet " "	2 5 gm meat extract, 150 cc water at 10 25 a m
10.80	" " Slight movement	Basal metabolism
10 67	Quiet "	8 gm glucose, 150 cc water at 10 24 a m

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THE ANTISCORBUTIC VALUE OF THE BANANA.

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The nutritive value of the banana as determined by its adequacy for the growth or maintenance of albino rats has recently been the subject of investigation by Sugiura and Benedict (1) Rats maintained on banana alone failed to increase in size and died after periods of 16 to 80 days without marked loss in weight It was suggested that the physical properties of the banana might not be favorable for the utilization of the food materials in the digestive tract of the rat. Further experiments in which the banana supplied only a part of the ration indicated that the banana was lacking in adequate amounts of protein and the water-soluble accessory (water-soluble B). For the cure of infantile scurvy the banana has been observed to have little antiscorbutic value (2), being decidedly inferior to the juice of the potato or orange. No studies of the antiscorbutic value of the banana in experimental scorbutus of the guinea pig have been reported.

Three cases of scurvy developed in the group of rats on an exclusive banana diet in the experiments of Sugiura and Benedict (1). No clinical or postmortem observations are recorded, however. The rat is not commonly considered to be susceptible to experimental scurvy, and McCollum and Pitz (3) have shown that normal growth and reproduction are possible in the rat on diets which produce scurvy in the guinea pig. Similar results have been obtained by Cohen and Mendel (4). Harden and Zilva believe that the antiscorbutic factor is necessary for the normal growth of the rat (5) even though those animals do not develop the typical lesions generally associated with the disease. Like conclusions have been reached more recently by Drummond (6). Tozer (7) has also reported that in guinea pigs on

diets deficient in factors other than the antiscorbutic principle, notably the fat-soluble accessory, histological study may reveal a condition closely resembling that present in experimental scurvy In view of these facts and of the absence of detailed clinical or autopsy data, it may be considered questionable whether Benedict and Sugiura were dealing with typical experimental scurvy in their rats.

The present series of experiments is concerned with the value of the banana as an antiscorbutic in experimental scurvy of the guinea pig.

EXPERIMENTAL.

The experimental animals were young guinea pigs, for the most part about 300 gm. in weight. The greater number of the animals were purchased from a local dealer, who bred small numbers of them, and in whose pens the sanitary conditions were known to be excellent   In later experiments it became necessary to purchase some animals from wholesale dealers. In all cases, however, the animals were kept under careful observation in the laboratory on mixed diets until it was established that they were in good health   The animals were confined in cages about 30 by 18 inches in dimension which allowed a reasonable amount of exercise   These cages were cleaned daily, steamed out with live steam on alternate days, and once a week were washed out with a weak solution of phenol   Food consumption was determined by daily weighing of the uneaten portion   With the exception of a few days at the outset of the experiments, the containers for the drinking water were sterilized daily   The guinea pigs were weighed on alternate days.

The symptoms which were taken as a criterion of the onset of scurvy were those described by Chick, Hume, and Skelton (8), and by Cohen and Mendel (4)   In all cases, the clinical data were checked up by postmortem examination, although it was not possible to secure histological examination of the tissues

Bananas as the Sole Source of Food —Sugiura and Benedict (1) have shown that the banana is deficient in protein and water-soluble B, and that adequate nutrition of the white rat is impossible on a diet consisting solely of bananas   Inasmuch as the nutritive requirements of the guinea pig have not been worked

out in such detail as have those of the white rat, it was considered desirable to study the influence of the banana diet on the growth of young guinea pigs Two typical experiments are presented in Chart 1. Guinea Pig K with an average daily consumption of over 65 gm of banana lost weight rapidly and died on the 26th day. No clinical signs of scurvy were evident and the postmortem examination of the animal revealed a marked condition of inanition, but no lesions of scurvy. Similar results
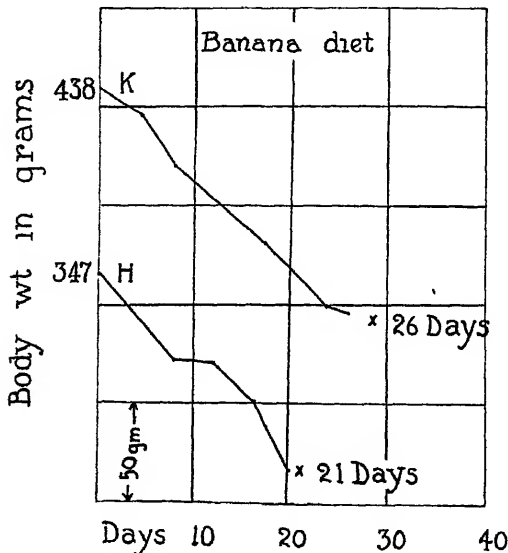
CHART 1 Guinea Pigs H and K received only ripe bananas, the average daily consumption being slightly over 50 and 65 gm respectively Both guinea pigs lost weight rapidly and died in 21 and 26 days respectively No scorbutic symptoms were present at any time

were obtained with Guinea Pig H whose average daily food consumption was somewhat less. The rapid decline in weight with no accompanying symptoms of scurvy was also shown in experiments (Chart 3) in which after a period of marked loss in weight the banana diet was supplemented by the addition of oatmeal. These results are similar to those reported by Sugiura and Benedict in experiments on white rats, although in the latter the decline in weight was less marked than in the present series.

Lack of the antiscorbutic factor can hardly be included in the list of the deficiencies of the banana as a foodstuff, since despite the marked malnutrition, as evidenced not only by failure of
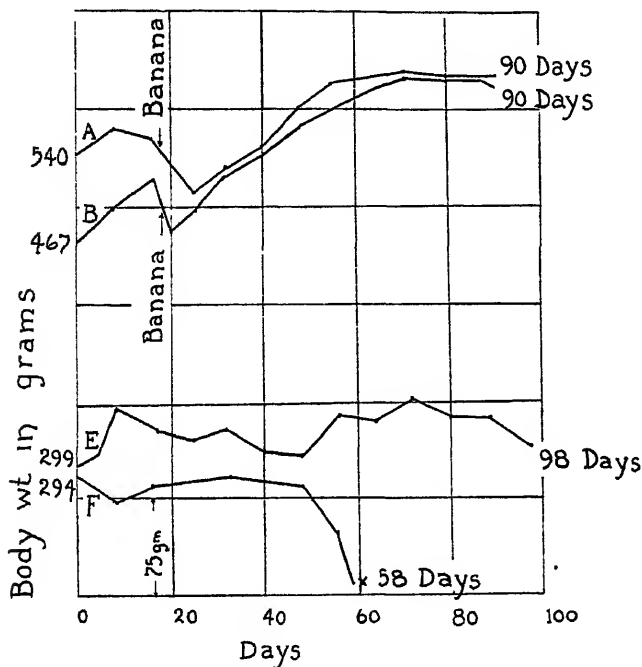
CHART 2 Guinea Pigs A and B received rolled oats *ad libitum* for 18 and 19 days respectively At this point tenderness of the joints was evident and loss of weight was marked Banana (35 gm) added to the diet relieved the scorbutic symptoms and permitted slow growth The animals were killed after 90 days No signs of scurvy were observed on autopsy Guinea Pigs E and F received rolled oats *ad libitum* and 35 and 25 gm of banana respectively No scorbutic symptoms developed The oat intake of Guinea Pig F gradually diminished and the animal died after 58 days Guinea Pig E was killed at the end of 98 days Autopsy findings were normal in both cases

growth, but even of maintenance on this diet, no scorbutic symptoms were observed

Banana and Rolled Oats.—The early experiments of Holst and Frolich (9) and later investigations from other laboratories (3, 4,

10, and 11) have shown that guinea pigs on an exclusive cereal diet usually die in from 20 to 30 days with scorbutic symptoms. In Chart 2 and Table I are presented typical protocols of experiments in which banana supplemented the oatmeal diet. In the earlier
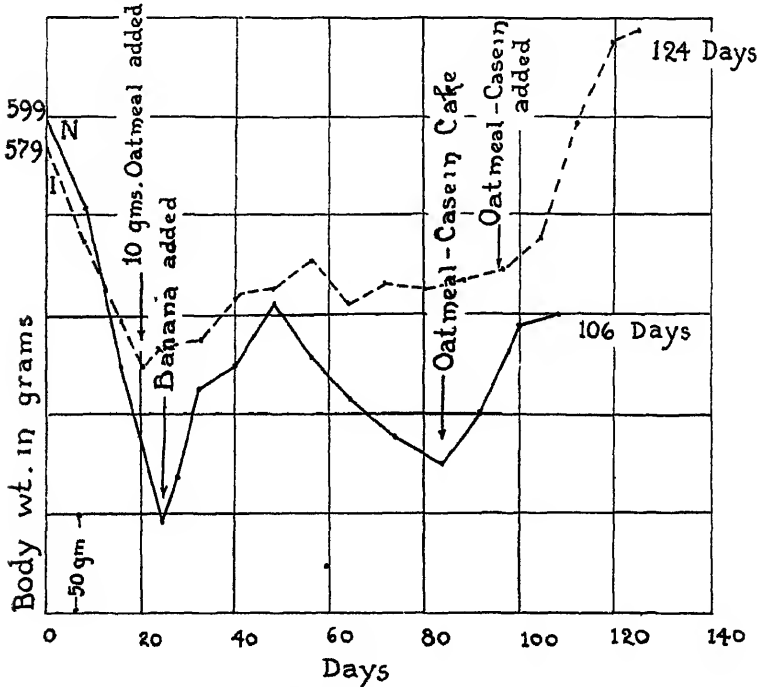
CHART 3   Guinea Pig I received an exclusive banana diet up to the 20th day, 10 gm of oatmeal were then added until the 93rd day, after which the oatmeal-casein cake was fed in place of the oatmeal   The animal was free from clinical symptoms of scorbutus throughout the period of the experiment   Guinea Pig N received rolled oats only for 24 days Soreness and slight enlargement of the joints developed about the 18th day   30 gm of banana as a supplement to the diet relieved the scorbutic symptoms although satisfactory growth was not made   When the oatmeal-casein cake was substituted for the rolled oats, rapid growth ensued

part of the experiments bran was also added to the diet, but since the animals chose the rolled oats and left the bran unconsumed its use was discontinued. Provided the daily intake of banana could be maintained at 25 gm. or more, no scorbutic

TABLE I

Guinea pig No	Duration	Weight			Diet	Notes
		Initial	Maximum	Final		
	days	gm	gm	gm.		
A	90	540	615	608	Oatmeal to 18th day, oatmeal and 35 gm banana thereafter	Marked loss in weight 12th to 18th day, slight gain to 60th day, no gain thereafter Killed No scurvy
B	90	467	604	592	Oatmeal to 19th day, oatmeal and 35 gm banana thereafter	Marked decline in weight 14th to 20th day, gradual gain to 56th day Killed No scurvy
C	26	687	693	483	Oatmeal	Marked loss in weight after 18th day Died Scurvy.
D	34	320	331	238	Oatmeal and 10 to 15 gm banana daily, banana eaten in small amounts only after 24th day	Marked loss in weight after 20th day Died Severe scurvy.
E	98	295	358	317	Oatmeal and 35 gm banana	No growth, fluctuations in weight Ate little food after 90th day Killed No scurvy.
F	58	290	299	213	Oatmeal and 25 gm banana, diminished intake of oats toward end of experiment	Progressive loss in weight with diminished food intake Died. Inanition No scurvy
G	44	329	329	255	Oatmeal and 20 gm banana	Prolapse of rectum Killed Marked impaction of cecum Mild scurvy
H	21	347	364	247	Banana about 50 gm daily	Died Inanition No scurvy
K	26	438	438	326	Banana about 65 gm daily	" " " "
I	124	579	641	641	Banana alone to 20th day, oatmeal and banana to 93rd day, oatmeal-casein cake thereafter	Marked loss in weight on banana diet, maintenance on banana-oat diet, vigorous growth on banana and oat cake
M	73	754	754	416	Banana alone to 20th day, oatmeal and banana to end of experiment little banana eaten last 2 weeks of experiment	Died Marked impaction of cecum Severe scurvy

TABLE I—*Continued*

Guinea pig No	Duration	Weight			Diet	Notes
		Initial	Maximum	Final		
	days	*gm*	*gm*	*gm*		
N	106	599	643	505	Oatmeal to 24th day, oatmeal and 30 gm banana to 75th day, oat cake and 30 gm banana thereafter	Loss in weight on oat diet, slight soreness of joints on 18th day, maintenance and slight growth on oat-banana diet, good growth on banana and oat cake Killed No scurvy
S	86	235	431	423	Oatmeal and 25 gm banana to 30th day, oat cake and 20 to 25 gm banana thereafter	Maintenance and slow growth on oat-banana diet, better growth on oat cake and banana Killed No scurvy
T	48	308	308	157	Oatmeal to 12th day, oatmeal and 25 gm banana to 30th day, oat cake thereafter	Died Marked clinical and postmortem symptoms of scurvy
U	48	270	323	198	Oatmeal to 18th day, oatmeal and banana to 30th day, oat cake and banana to 36th day oat cake alone thereafter	D ed Severe scurvy
V	86	272	491	491	Oatmeal to 18th day, oatmeal and banana to 29th, oat cake and 15 gm banana thereafter	Rapid gain in weight after addition of oat cake and banana No scurvy
W	86	276	477	477	Oatmeal to 20th day, oatmeal and banana to 28th day, oat cake and 25 gm banana thereafter	Loss in weight on oats alone, slight gain on banana-oat diet, rapid gain on oat cake and banana
Y	24	352	352	223	Oat cake	Rapid decline in weight after 16th day Died Scurvy
Z	23	517	544	315	" "	Marked loss in weight after 14th day Died Mild scurvy
10	81	230	500	495	Oat cake and 10 gm banana	Steady growth In excellent condition at end of experiment

TABLE I—*Concluded*.

Guinea pig No.	Duration	Weight			Diet	Notes
		Initial	Maximum	Final.		
	days	*gm*	*gm*	*gm*		
11	83	238	335	332	Oat cake to 23rd day, oat cake and slightly less than 10 gm banana thereafter, in last 10 days banana intake slightly above 10 gm.	Decline from 13th to 23rd day; increase to 39th day, decline to 49th day, steady gain thereafter Soreness of joints from 30th to 67th day. In excellent condition at end of experiment.
12	72	258	433	433	Oat cake and 15 gm banana	Fair growth, no scurvy
13	72	338	476	476	Oat cake and 15 gm. banana	Fair growth, marked increase in weight during last 12 days No scurvy
14	72	273	518	518	Oat cake and 20 gm banana	General but somewhat irregular growth to 42nd day Regular growth thereafter.
16	72	443	594	594	Oat cake and 20 gm banana	Fair growth No scurvy.

symptoms were observed.   Ingestion of quantities of banana less than this failed to give satisfactory protection against scurvy (Table I, Guinea Pigs D, G).   Adult animals (Chart 2, Guinea Pigs A, B) maintained their weight or grew slowly, while younger animals (Chart 2, Guinea Pigs E, F) were barely able to maintain themselves, and in some cases showed marked decline in body weight, but no scorbutic symptoms, either clinical or postmortem. These results would seem to indicate that the banana is greatly inferior to most other fruits and vegetables as an antiscorbutic.

Inasmuch as the oat has been shown (3) to be deficient in the protein, inorganic salt, and fat-soluble A factors, and as the banana is also deficient in at least one of these same factors, it was felt that although the experiments had demonstrated a definite antiscorbutic value of the banana, the results could hardly be judged on the quantitative basis in view of the above mentioned deficiencies of the diet   A greater amount of the anti-

scorbutic principle may be requisite when the diet is not other-
wise adequate to provide for normal growth and maintenance.
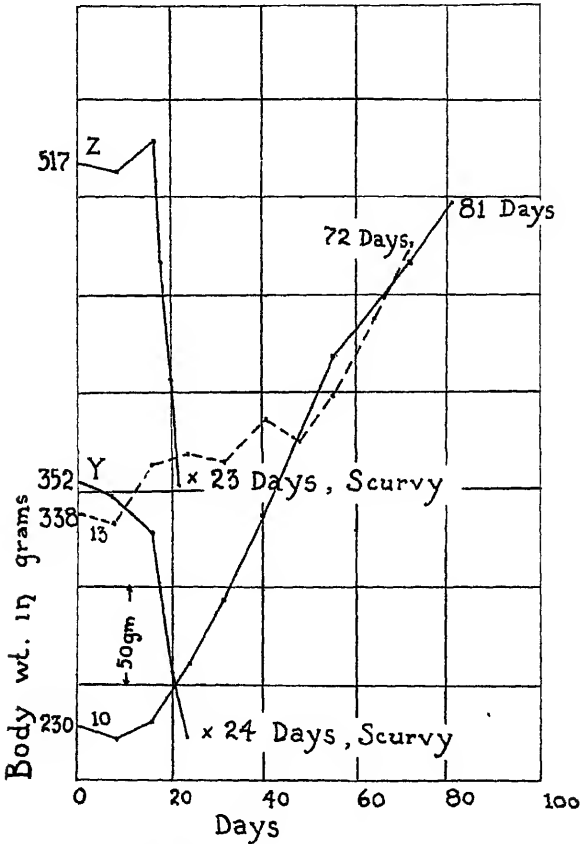
CHART 4 Guinea Pigs Y and Z were maintained on the oatmeal-casein
cake without the addition of banana, and developed scurvy which resulted
in death   Guinea Pigs 10 and 13 received the same diet with a supple-
ment of 10 and 15 gm of banana daily   The animals were in excellent
condition throughout the course of the experiment.

Banana and Oatmeal-Casein Diet.—For the purpose of render-
ing the basal diet more nearly adequate for normal growth, the
following ration was prepared

Autoclaved rolled oats	500 gm
Wheat bran	100 "
Purified casein	40 "
Raw milk	200 cc
Calcium lactate	15 gm
Sodium chloride	15 "
Distilled water	400 cc

The oats were heated in an autoclave for 30 minutes at 120°. The dry ingredients were thoroughly mixed, the liquids added, the pasty mass was spread out in thin sheets, and dried at 75–80° The resulting cake was relished by the animals so that little difficulty was experienced in securing a daily intake of 20 gm.

Guinea pigs maintained on this oatmeal cake developed scurvy as rapidly as on an exclusive oatmeal diet (Chart 4, also Guinea Pigs T, U. Table I)   When this cake was supplemented by banana, not only was scurvy prevented, but rapid growth in young animals also occurred, growth which was in striking contrast to the results observed when banana supplemented an exclusive oat diet   This contrast is clearly shown in Chart 3   With this more complete diet protection against scurvy and normal growth were obtained with 10 to 15 gm of banana   Thus Guinea Pig 10 (Chart 4) on a diet of 20 gm. of oat cake and 10 gm of banana daily gained 265 gm. in 81 days, increasing in weight from 230 to 495 gm   Similar results were obtained with the other animals of this group (Guinea Pigs 10 to 16, Table I).   It is evident that when supplementing a diet otherwise adequate, the banana has considerable value as an antiscorbutic, although its potency in this respect is not so great as that of the orange or potato.

SUMMARY

1. Guinea pigs fed on an exclusive diet of bananas are unable to maintain their body weight and die in 20 to 30 days.   Autopsy reveals a condition of marked inanition, but no lesions character-istic of scurvy.

2. Bananas in amounts greater than 25 gm daily as a supple-ment to a diet of rolled oats prevent the onset of scurvy.   Such a diet, however, does not permit normal growth in young animals Less than 25 gm of banana as a supplement to the oat diet does not protect against scurvy.

3. Scurvy can be readily produced experimentally on a diet of autoclaved rolled oats supplemented by bran, milk, casein, and inorganic salts When such a diet is further supplemented by banana, 10 to 15 gm. will serve to protect against scurvy. Such a diet not only affords protection against scurvy but results in rapid growth of young guinea pigs

4. These experiments suggest that a lower content of the antiscorbutic principle may be sufficient to protect against scurvy if the diet is adequate in its content of the other essential dietary constituents.

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THE DETERMINATION OF CARBON MONOXIDE IN BLOOD.

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The determination of carbon monoxide in blood may be performed by gasometric, colorimetric, or spectrophotometric methods.

Previous gasometric methods have been laborious and most of them required special apparatus (as those of Gréhant, de Saint Martin, Nicloux, and others). The method employed by Zuntz and Plesch (1) seems to be the easiest. They determine the carbon monoxide in 1 cc. of blood, using ferricyanide to set the gases free (CO and O_2) and burn the CO to CO_2, which is absorbed by KOH. They calculate the amount of CO by the difference in pressure as in Haldane and Barcroft's method for blood gas analysis According to the authors, the expulsion of the blood gases is finished after 1 hour.

The colorimetric method of Haldane (2) is based upon the fact that dilute carmine solutions have nearly the same color as dilute solutions of carbon monoxide hemoglobin. If it is known how much carmine has to be added to normal blood in order to give it the same color as blood completely saturated with carbon monoxide, and it is determined how much of the carmine must be added to normal blood to give it the color of the blood with the unknown content of CO, a simple calculation by proportion will give the degree of saturation with carbon monoxide.

This method seems to be very accurate in Haldane's hands, while others, like Krogh (3), and Zuntz and Plesch (1), have been unable to obtain good results with it. Plesch (4) has modified it, using a hemoglobin solution saturated with carbon monoxide instead of carmine for the titration.

Spectroscopic methods have been used by Hufner and later by Hartridge (5). Krogh (6) has recently modified the method of the latter and used it for the determination of the oxygen capacity of very small amounts of blood; his method is still rather rough, but he hopes to make it more accurate with finer instruments

An easy gasometric method seems still to be needed and we have worked out one which permits the determination of the carbon monoxide gasometrically in 2 cc of blood in the course of 10 to 15 minutes   We have employed this method for the determination of the blood volume and for the study of the action of carbon monoxide on blood

The Method.

The principle of our method is to set free the oxygen and carbon monoxide from their combination with hemoglobin in the blood by addition of ferricyanide and then to remove both gases with the help of a Torricellian vacuum in the Van Slyke apparatus for blood gas analysis   The oxygen is absorbed in the apparatus by alkaline pyrogallate and the volume of residual carbon monoxide is measured directly at atmospheric pressure, a correction being made for the small and constant amount of nitrogen gas physically dissolved by blood

The procedure is, up to the time when the expelled gas is measured, exactly the same as that for the oxygen method described by Van Slyke (7), and it is therefore unnecessary to repeat it here, the same amount of blood and the same solutions are used, and only the shaking has to be continued a little longer before a constant reading is obtained   This takes about 2 to 3 minutes and is a little different for different species of blood; it probably depends upon the facility with which the blood is laked

When the reading of the volume of the gas mixture, consisting of oxygen, carbon monoxide, and a little nitrogen, is constant, a solution of alkaline pyrogallate[1] is introduced into the cup of the apparatus, is covered by a thin layer of paraffin oil, and is allowed to flow slowly down the inner wall of the graduated part of the

---

[1] Prepared by dissolving 10 gm of pyrogallic acid in 200 cc of strong potassium hydroxide (160 gm of KOH dissolved in 130 cc of water)

apparatus   A little suction is produced during this part of the procedure by lowering the leveling bulb slightly

The absorption of the oxygen is very rapid and is completed in less than 1 minute, the reading is taken and the pyrogallate solution introduced once more until a constant reading is obtained. The gas is then measured under barometric pressure in the same way as described by Van Slyke for carbon dioxide (8) and oxygen.

As the solution is very dark and it is a little difficult to get good readings of the meniscus, we have produced a new meniscus by letting a little water flow down after the pyrogallate solution, the water floats on the top of the fluid and one can get readings to about 0.002 cc   Instead of water a few drops of octyl alcohol may be used.

The apparatus is washed out twice with dilute ammonia solution after each determination.

Calculation —The gas measured is reduced to standard conditions by multiplying by the factor $(0\ 999-0.0046\ t) \times \dfrac{\text{Barometer}}{760}$, t being the temperature in °C.   If 2 cc of blood have been used, the values of this factor in Column 3 of Table I of Van Slyke's paper on oxygen[2] may be used, the result then being expressed in cc. of CO per 100 cc. of blood, when the nitrogen correction, 1.2 cc., is subtracted [3]

EXPERIMENTAL.

Air was analyzed in the Van Slyke apparatus in order to find the best way of absorbing the oxygen   When the pyrogallate solution was introduced in the manner described above, the oxygen was absorbed in 30 seconds

The method was tried in the following way.   As the oxygen and the carbon monoxide replace each other in the combination with hemoglobin and the oxygen capacity equals the carbon monoxide capacity, blood with known percentages of carbon monoxide may be obtained by mixing different amounts of blood

[2] Van Slyke, D D , *J Biol Chem* , 1918, xxxiii, 130
[3] The nitrogen correction is 1 2 per cent, instead of the calculated value 0 9 per cent, when actually determined by Bohr and by ourselves

saturated with carbon monoxide with blood saturated with air. By taking, for instance, one part of CO blood and four parts of O_2 blood, the analysis should give 20 per cent CO and 80 per cent O_2.

Table I shows the amounts of oxygen and carbon monoxide found by analysis and the amount calculated.

TABLE I

No	Blood used	Found			Calculated			Kind of blood
		Oxygen	CO	Proportion of hemoglobin saturated with CO	Oxygen	CO	Proportion of hemoglobin saturated with CO	
	cc	cc	cc	per cent	cc	cc	per cent	
1	2	0 212	0 206	50 7	0 209	0 209	50 0	Guinea pig.
2	2	0 208	0 210	49 8	0 209	0 209	50 0	Same blood.
3	2	0 118	0 240	32 9	0 117	0 238	33 3	Rabbit
4	2	0 170	0 328	34 1	0 116	0 332	33 3	Ox
5	2	0 088	0 250	26 0	0 084	0 252	25 0	Rabbit
6	'	0 128	0 390	24 5	0 129	0 388	25 0	Ox.
7	2	0 068	0 240	22 1	0 062	0 246	20 0	Rabbit.
8	2	0 054	0 224	19 43	0 046	0 231	16 7	"
9	4	0 091	0 434	17 3	0 088	0 437	16 7	Same blood.

It is seen from Table I how closely the found values agree with those calculated, except in No. 8, where the discrepancy is 2.7 volumes per cent. By taking 4 cc. of the same blood instead of 2 cc., this error is brought down to 0.6 volume per cent. We therefore recommend the use of a little more blood, 3 or 4 cc. (and the correspondingly increased amount of ammonia), for analysis, if the percentage saturation of CO is very small and the actual amount of CO found in 2 cc. of blood is 0.05 cc. or less.

In two rather rough experiments, two guinea pigs were given illuminating gas under a bell jar until they fell unconscious They were then taken out and blood was drawn by heart puncture for analysis. One of them died while the blood was being taken; the other recovered and behaved normally an hour afterwards.

Table II shows that a guinea pig can recover after carbon monoxide poisoning, even when the blood is 76 3 per cent saturated with carbon monoxide.

TABLE II

	O$_2$	CO	Saturation with CO	Remarks
	cc	*cc*	*per cent*	
Guinea Pig 1	0 115	0 353	75 4	Died during bleeding.
" " 2	0 080	0 258	76 3	Recovered

SUMMARY.

A method is described for the determination of carbon monoxide in blood, the technique of which is exactly the same as that previously described by Van Slyke for the determination of oxygen, except that after the gases are extracted the oxygen is absorbed in the apparatus by introducing alkaline pyrogallate solution. The carbon monoxide remains and is measured directly at atmospheric pressure.

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THE DETERMINATION OF BLOOD VOLUME BY THE CARBON MONOXIDE METHOD.

By HARALD A SALVESEN

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There is still much confusion regarding the exact value of the blood volume in human beings, though much work has been done in this field of investigation   Values for the blood volume for men are given, ranging from $\frac{1}{21}$ to $\frac{1}{8}$ of the body weight. These widely differing results have been obtained by the use of different methods.

There are one direct and several indirect methods for the determination of the blood volume.

Direct method —Welcker (1) in 1854 was the first one to determine the blood volume by a method which still is regarded as the standard, he bled animals to death, washed out the vessels with water, and extracted the hemoglobin still remaining in the tissues by mincing the organs minus the bile and the content of the bowels, and placing them in water for several days   By comparing the hemoglobin content of the first blood and the blood washings and extracts brought together, he found the blood volumes of mammals to constitute $\frac{1}{13}$ of the body weight   The same value was obtained for human beings by Bischoff (2), who used this method on two criminals

Welcker's method has been modified and improved by several investigators, but the general principle is the same as in 1854   The results obtained in animals with this method have differed because of incomplete washing and extraction, and the use of inexact methods for the hemoglobin determinations

Indirect methods —These methods must be used in experiments on living animals   There are various principles for the indirect determination of the blood volume   The best of them may be divided into two groups (1) A known amount of an easily determinable substance, which is kept within the circulatory system for a sufficiently long time for thorough mixing, is introduced into the blood and the concentration of it deter-

mined   (2) The blood is either (a) diluted, or (b) concentrated in various ways, and the blood volume calculated from the variation in the content of hemoglobin or corpuscles

Group 1 (a) Carbon Monoxide Method —The principle of this method is to administer a certain amount of carbon monoxide gas to the individual, and then to determine the degree of saturation of the blood with CO or the actual amount of CO per unit of blood   It was first used by Gréhant and Quinquaud (3), and later by Haldane and Smith (4), Oerum (5), Douglas (6), Boycott and Douglas (7), and Plesch (8)   (b) The dye method of Keith, Rowntree, and Geraghty (9) is the method most widely used for the present in clinical work   A dyestuff, vital red, is injected intravenously and determined colorimetrically in the blood   The relative amounts of plasma and corpuscles are determined by the hematocrit method   (c) The antitoxin method of von Behring (10) is based upon the observation that tetanus antitoxin remains in the circulation for a long period, a known amount is injected, and the concentration then determined in the blood   (d) The acacia method of Meek and Gasser (11) has been tried only in animals so far, and the experience is not large enough to judge of its utility   Acacia is injected and determined in the blood as furfurolphloroglucine

Group 2 (a) Dilution Methods.—The only one of these methods which has withstood criticism is based upon the observation of Cohnstein and Zuntz (12) that isotonic sodium chloride solutions are kept in the circulation for a relatively long time, and diffuse very slowly out into the tissues, this is utilized for the determination of the blood volume, the red cells being counted before and after infusion. Plesch (8) seems to have developed this method to further exactness by determining the hemoglobin instead of the cells with the help of his chromophotometer   As the variations in the cell or hemoglobin content obtained by dilution hardly exceed 10 per cent, the exactness of the method evidently depends on how accurately these constituents of the blood can be determined.

(b) Concentration Methods.—The method of Tarchanoff (13), who determined the hemoglobin before and after a steam bath and the decrease in weight through loss of water, and calculated the blood volume from these two factors, has been justly criticized and cannot be relied upon, as the water may be derived from other sources in the body than the blood

Quincke (14) transfused blood with a certain amount of red corpuscles to two anemic patients and calculated the blood volume from the increase in the red count   Lindeman (15) uses the same principle   These methods can only be used in anemia.

Of these methods only three are of practical value for physiological and clinical purposes, they are the carbon monoxide method, the infusion method of Cohnstein and Zuntz, and the vital red method.

The results obtained by these methods vary   In animals, both the carbon monoxide method and the infusion method have given nearly the same values as the Welcker method, Gréhant and Quinquaud (3) found in nine dogs values from $\frac{1}{11}$ to $\frac{1}{13\ 8}$ of the body weight with the CO method,

which correspond to Welcker's own results in dogs   Douglas (6) found a close agreement between the CO method and the bleeding method in five rabbits, Boycott and Douglas (7), repeating the experiments later, found a little higher value with the CO method (2 per cent)   Plesch (8), in dogs, tried subsequently the CO, the infusion, and the bleeding method, and the results were uniform   The vital red method has never been checked up by the Welcker method as far as can be seen from the literature

In human beings the results are widely different   Bischoff's values, $\frac{1}{13}$ of the body weight, were regarded as the standard until Haldane and Smith (4), with the CO method and carmine titration, in fourteen normal men found $\frac{1}{21}$, the highest value being $\frac{1}{16}$ and the lowest $\frac{1}{30}$ (in a very fat man).   Oerum (5) using the same technique, found in men $\frac{1}{19\ 2}$, and in women $\frac{1}{21\ 8}$ Plesch (8), using a gasometric method for the CO determination in four men, found the average ratio $\frac{1}{17\ 9}$, also in five persons, some of whom were reported to be fat, he determined the blood volume with the infusion method and found $\frac{1}{19\ 1}$ Bischoff's results, therefore, seem to be too high, inasmuch as the two criminals were hardly normal individuals, one at least suffering from scurvy   The method used is also open to criticism

Douglas (16), in 1910, made a series of determinations with the CO method on himself and another subject with Haldane's technique, but waited a longer time before he took the blood sample for analysis   These results show a mean value of $\frac{1}{13\ 9}$ for his own and $\frac{1}{12\ 5}$ for the other subject, values more in accordance with those of Bischoff   He found errors in Haldane and Smith's determinations due to incomplete mixing of the blood, as the blood sample was taken too early after the breathing of the carbon monoxide.

In 1915 Keith, Rowntree, and Geraghty (9), with their vital red method, found still higher values, the mean in normal men being $\frac{1}{11\ 8}$ of the weight This method has never been controlled by the Welcker method as far as can be seen, but the authors show its reliability toward relative changes by drawing a certain amount of blood and finding a corresponding drop in the blood volume

It may be seen from this review of the literature how uncertain is our knowledge of the blood volume in human beings.   Since the carbon monoxide method in animals has given satisfactory results as compared with the standard method of Welcker, and

in human beings the results obtained by the various investigators have differed widely, it seems worth while to make further investigations in this field, especially since the technique used before has been rather difficult

It seems certain, according to Douglas, that Haldane and Smith's figures are too small, and so must be the figures of Oerum, as he used the same technique. There remain therefore,. only the determinations of Plesch in four persons, giving the average of $\frac{1}{17.9}$ and those of Douglas, on himself and another man, giving the values of $\frac{1}{13.9}$ and $\frac{1}{12.5}$ of the body weight.

The carbon monoxide method has been criticized by Dreyer (17) and his coworkers, who, in rabbits, used Haldane and Smith's technique, and got so much divergence in the figures that they concluded it could not be used in its present form. They therefore determined the blood volume by injecting in rabbits' blood a known amount of agglutinin, determined the percentage in the serum, then washed out the circulation, and determined the percentage of agglutinin in the washing They claim the blood volume to be a function of the surface area, so that, for instance, smaller rabbits have a relatively higher blood volume than the larger ones.

The adverse criticism of the carbon monoxide method may be due to the difficulty of the technique, as the carmine titration of Haldane requires long training and a highly developed color sense. All the carbon monoxide determinations in the present paper are performed with the help of the gasometric method, described in the preceding paper, which makes the whole technique much simpler and fitted for general use.

Bood Volume Determinations in Animals.

The results of numerous determinations of Boycott and Douglas (7) show that rabbits' blood constitutes from $\frac{1}{22}$ to $\frac{1}{18.1}$ of the body weight, as determined both with the carbon monoxide and the washing out method. The average of 52 rabbits with the washing out method was $\frac{1}{20.9}$, or 4 77 cc. of blood per 100

gm. of body weight, while the carbon monoxide method (with the carmine titration) gave a little higher result, $\frac{1}{18.1}$ or 5.5 cc. per 100 gm.

Methods

The arrangement used was that described by Douglas (6). It is therefore unnecessary to repeat it here.

The principle is to let the rabbit breathe into a closed system which is supplied with arrangements for removing the carbonic acid and renewing the oxygen. A measured amount of carbon monoxide is introduced into the apparatus, and 10 minutes after the entire amount is given a sample of blood is drawn from the ear vein and analyzed for carbon monoxide. At the same time a sample of the air in the chamber is taken for determination of the O_2, CO_2, and CO. The blood volume is calculated from the amount of carbon monoxide absorbed by the animal and the concentration of it in the blood. As in Douglas' experiments, a tube connected with a bell jar, partly immersed in a glass of water, was introduced into the respiratory chamber, and served as an indicator of the pressure in the apparatus. While the air sample was drawn, the oxygen current was cut off and the water allowed to rise in the bell jar by raising the glass in order to compensate for the negative pressure produced by the sucking out of the air.

The capacity of the apparatus (the chamber and the air in the rubber tubing, the pump, and the bell jar) was 750 cc., a little larger than that of the apparatus employed by Douglas (6), and Boycott and Douglas (7).

The carbon monoxide was prepared by heating oxalic acid and concentrated sulfuric acid, and the gas was collected over water made alkaline with sodium hydroxide, with which the gas was shaken in order to get rid of the CO_2. The gas was analyzed every 2nd day by shaking it in a Hempel pipette with cuprous chloride solution, which absorbs the carbon monoxide and O_2, and from the amount of nitrogen left the air content of the gas was calculated. The gas first evolved was discarded. The carbon monoxide content of the rest was 95 to 98 per cent.

The carbon monoxide in the blood was determined by the method described in the preceding paper, the blood, 4.5 cc. in

all, being drawn from the ear vein without stasis and kept under paraffin oil, 2 cc were used for each analysis. The hemoglobin was determined by the Palmer method (18)   The air in the chamber was analyzed for O_2 and CO_2 in the Haldane-Henderson apparatus.

The determination of the carbon monoxide left in the chamber could not be made by gasometric methods, and the method of Haldane (19) was employed, the principle of which is to shake the carbon monoxide-containing air with blood, to estimate colorimetrically the percentage of saturation of the blood with carbon monoxide, and deduce from this value, and the percentage of O_2 present, the percentage of carbon monoxide in the air   As bloods of different species and also of different individuals show different dissociation curves for the carbon monoxide hemoglobin, as shown by Krogh (20) and Haldane (21), blood from a single sheep was used, in which the dissociation curve was previously determined (preceding paper).   The dissociation curve is a hyperbola of the formula

$$\frac{(O_2 \text{ percentage in air})}{(CO \text{ percentage in air})} \times \frac{Hb\ CO}{Hb\ O_2} = K$$

In the blood used $K = 179$ at $24°$

For the determination of the dissociation curve of the blood of our sheep, 5 cc samples of the blood were rotated in bottles of known capacity (approximately 1 liter), filled with air plus known amounts of carbon monoxide   The blood was first placed in the bottle, which was then closed by a stopper containing a three-way capillary cock   A known volume (1 to 4 cc) of analyzed CO gas was then forced in from a micro-gas-burette, in which the volume delivered could be read over mercury to within 0.002 cc   The tubes of the cock were filled with the CO before the measured amount was admitted into the bottle, so that errors due to dead space were avoided   The bottle with the blood and gas mixture was rotated for 2 hours at 24°C.   Trial showed that equilibrium was obtained in this time.   Samples of 2 cc. of blood were then withdrawn and used for the determination of O_2 and CO as described in the foregoing paper.   The volume of CO taken up by the 5 cc. of blood was subtracted from the volume of CO originally added, in order to estimate the amount left in the gas

phase. The oxygen concentration remained that of atmospheric air.

The results obtained in four determinations are shown in the curve of Fig. 1. The curve is the hyperbola plotted from the formula, with 179 taken as the value of K; the crosses represent the results experimentally obtained.
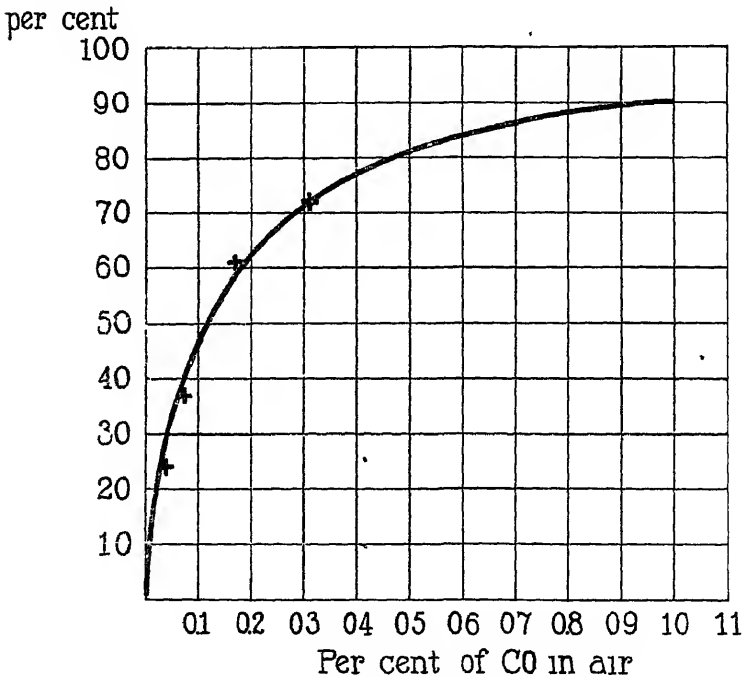
per cent

Fig 1 The curve is the hyperbola plotted from the formula, with 179 taken as the value of K, the crosses represent the results experimentally obtained

The value of K being known, small percentages of CO in air could be ascertained by shaking the latter with a known volume of the blood and determining the Hb O_2 and Hb CO, the calculation being *per cent CO in air* $= \dfrac{21\,9}{179} \times \dfrac{\mathrm{Hb\,CO}}{\mathrm{HbO_2}}$. In calculating the actual percentage of CO in the gas-sampling tube allowance was made for the amount of CO taken up by the blood with which the air was shaken The colorimetric determination was

made by the method employed by Plesch (8), which is very simple
and which gave fairly good results when used on blood with
known percentages of CO.   The principle is: Three samples of
the same blood are saturated; No. 1 with air, No. 2 with CO,
and No. 3 with the air containing the unknown percentage of CO.
0.05 cc. of each sample is diluted with 10 cc. of a 1 per cent solu-
tion of Na_2CO_3 in each of three small test-tubes of equal bore.
No. 2 is added to No. 1 until the color is the same as in No. 3.
If, for instance, equal color is obtained by adding 2.5 cc. of No. 2
to 5 cc. of No. 1, then the degree of saturation is $\dfrac{2.5}{2\ 5+5} = 33.3$
per cent.

Results.

In fourteen rabbits taken from the stock the blood volume
was determined.   In two of the female rabbits the values obtained
were much higher than the average, and they later proved to be
pregnant.   They were placed in a separate group and the blood
volume determined again, post partum.   In some of the other
rabbits the blood volume also was determined twice.   The
results are given in Tables I to V.

TABLE I.

Normal Male Rabbits.

Rabbit No.	Date	Weight	Hemoglobin	Blood volume.	Relation to body weight	Cc per 100 gm
	1919	*gm*	*per cent*	*cc*		
77	July 3	1,500	(?)*	79 4	$\dfrac{1}{19\ 1}$	5 20
93	" 8	1,900	(?)*	95 7	$\dfrac{1}{19\ 9}$	5 02
89	" 10	1,600	93 4	80 9	$\dfrac{1}{19\ 8}$	5 05
80	" 10	2,250	94 3	107 1	$\dfrac{1}{21}$	4 76
99	" 14	3,010	86 2	146 9	$\dfrac{1}{20\ 5}$	4 88
82	" 14	2,200	87.7	115 4	$\dfrac{1}{19\ 06}$	5 25
98	" 15	2,200	93 4	111 1	$\dfrac{1}{19\ 8}$	5 05

* Determinations lost because of an incorrect hemoglobin standard

TABLE II.

Normal Female Rabbits

Rabbit No	Date.	Weight	Hemoglobin	Blood volume	Relation to body weight	Cc per 100 gm
	1919	*gm*	*per cent*	*cc*		
66	July 2	2,300	(?)*	122 2	$\frac{1}{18\ 8}$	5 30
76	" 7	1,250	83 3	50 8	$\frac{1}{24.6}$	4 06
88	" 9	1,960	86 2	100 6	$\frac{1}{19\ 4}$	5 14
83	" 11	2,630	76 9	129 2	$\frac{1}{20\ 3}$	4 93
87	" 11	2,320	51 4	127 5	$\frac{1}{18\ 2}$	5 49

* Determination lost because of an incorrect standard

TABLE III

Repeated Determinations
Male Rabbits

Rabbit No	Date	Weight	Hemoglobin	Total oxygen capacity	Blood volume	Ratio	Cc per 100 gm
	1919	*gm.*	*per cent*	*cc*	*cc*		
93	July 8	1,900	(?)*	(?)*	95.7	$\frac{1}{19\ 9}$	5 02
	" 21	1,940	59 3	10.14	92.4	$\frac{1}{21}$	4.76
77	" 3	1,500	(?)*	(?)*	79 4	$\frac{1}{19\ 1}$	5 20
	" 18	1,540	75 0	13 87	72 9	$\frac{1}{21\ 1}$	4 74
80	" 10	2,250	94 3	18.68	107 1	$\frac{1}{21.1}$	4 76
	" 17	2,270	90 9	17 54	102 1	$\frac{1}{22.2}$	4 50
99	" 14	3,010	86 2	21 08	146 9	$\frac{1}{20\ 5}$	4 88
	" 18	3,070	74 1	22 33	162 1	$\frac{1}{18\ 9}$	5 29

* Determination lost because of an incorrect standard

TABLE IV

Repeated Determinations
Female Rabbits

Rabbit No	Date	Weight	Hemo-globin	Total oxy-gen capacity	Blood volume	Ratio	Cc per 100 gm
	1919	*gm*	*per cent*	*cc*	*cc*		
76	July 7	1,250	83 3	7 83	50 8	$\frac{1}{24\ 6}$	4 06
	" 15	1,350	66 6	8 30	66 8	$\frac{1}{20\ 2}$	4 95
81	" 11	2,320	51 4	12 12	127 5	$\frac{1}{18\ 2}$	5 49
	" 17	2,300	47 4	11 44	129 0	$\frac{1}{17\ 8}$	5 62

TABLE V

Blood Volume in Pregnant Rabbits before and after Term

Rabbit No	Determination made	Date	Weight	Hemoglobin	Total oxygen capacity	Blood volume	Ratio	Cc per 100 gm	Remarks
		1919	*gm*	*per cent*	*cc*	*cc*			
65	Before	July 8	2,650	69 4	22 86	177 3	$\frac{1}{14\ 9}$	6 70	Young ones, July 15
	6 days post partum	" 21	2,290	76 3	15 24	108 3	$\frac{1}{19\ 4}$	5 14	
87	Before	" 9	2,560	94 0	24 29	141 1	$\frac{1}{18\ 1}$	5 52	
	"	" 16	2,570	74 4	22 2	161 3	$\frac{1}{15\ 3}$	6 54	Young ones, July 20
	11 days 'after	" 31	2,350	76 3	15 59	110 5	$\frac{1}{21\ 2}$	4 72	

In Tables III and IV is introduced a column, "total oxygen capacity," the figures in which indicate all the oxygen with which the blood is able to combine, calculated from the blood volume and the hemoglobin percentage In our hemoglobin standard 100 per cent equals 18.5 volumes per cent of O_2.

DISCUSSION.

The results are expressed in parts of the crude body weight, though this may vary for various causes, such as food intake, content of bowels, etc   The average value for the blood volume in seven male rabbits (eleven determinations, Tables I and III) is $\frac{1}{20.21}$ of the body weight, or 4.95 cc. per 100 gm.   The average in five non-pregnant female rabbits (seven determinations, Tables II and IV) is $\frac{1}{19\ 9}$ of the weight, or 5.02 cc. per 100 gm. If the two determinations from Table V in rabbits post partum are added, the mean for all seven females is the same, $\frac{1}{19.99}$, or 5 0 cc. per 100 gm.   The results are in accordance with those obtained by Boycott (7) and coworkers with the washing out method.

The repeated determinations in Tables III and IV show a fairly close agreement in some of the rabbits, while in Rabbits 99 and 76 there is a considerable difference.   But the total oxygen capacity is nearly constant in these two rabbits.   Rabbit 76 had increased in weight from 1,250 to 1,350 gm , and to get comparable values the oxygen capacity in the first determination has to be multiplied by $\frac{1,350}{1,250}$ = 1.08, and this gives the value of 8.46 cc which is very close to the value found the second time, 8.30 cc. The same phenomenon is seen in the pregnant rabbit, No 87, of Table V.   The two determinations before partus gave $\frac{1}{18.1}$ and $\frac{1}{15\ 3}$ of the weight, which is a large increase in the blood volume The hemoglobin concentration dropped, however, so that the oxygen capacity of the total blood supply of the animal remained constant, the blood merely having become diluted in the interval between determinations.   About a week after partus the blood volume was again normal

The blood volume of the rabbit, therefore, seems to be able to change normally, probably in the way that fluid passes in and out through the capillaries, a phenomenon in analogy with what has been observed in human beings in shock.   Rabbits 99 (Table III), 76 (Table IV), and 87 (Table V), in which these changes in

the blood volume and constancy of the total oxygen capacity were so marked that they cannot be due to exper mental error, show the smallest volume the first time, when they were unexperienced and scared when put into the apparatus, and a larger volume the second time, when they were used to the procedure. The material is too small, however, to draw any conclusion on this point, but Douglas has observed the same phenomenon of changes in the volume and constancy of oxygen capacity.

Table V shows the well known fact that pregnant animals have a laiger blood volume absolutely and relatively than non-pregnant. A week post partum the blood volume is restored to the normal value. ·

Blood Volume Determinations in Human Beings.

The arrangement of apparatus was nearly the same as that described by Haldane and Smith (4), except that an ordinary Wolff bottle, filled with sticks of potassium hydroxide,[1] was used for the absorption of the carbonic acid, as seen in Fig. 2, and the experiments lasted longer, the subjects breathing for 10 to 15 minutes after the entire amount of carbon monoxide was given. The oxygen was supplied from a cylinder as rapidly as was necessary in order to keep the amount of gas in the bag approximately constant. The estimation of the relative volume of air in the bag was facilitated by placing the bag horizontally with a scale behind it as an indicator of the degree of filling

The apparatus was filled with carbon monoxide to the three-way stop-cock before the experiment was started. The volume of the connecting parts and the bag, filled with air to the mark, was 3,700 cc For the calculation of the amount of carbon monoxide left the volume of the lungs must be added; this is about 3,000 cc., and the total volume of the air in which the carbon monoxide was distiibuted thus was 6,700 cc.

The blood, being assumed to constitute not less than $\frac{1}{19}$ of the body weight, the amount of carbon monoxide given to the sub-

[1] Potassium hydroxide was used because of the high solubility of the potassium caibonate formed during the experiment, this carbonate was washed out by rinsing the sticks with water now and then

ject was so calculated that the saturation of the blood with carbon monoxide would not exceed 20 to 25 per cent. For instance, with the body weight 70 kilos, hemoglobin 120 per cent, blood volume at least $\frac{70}{19}$ =3.7 liters, and as 100 per cent hemoglobin =
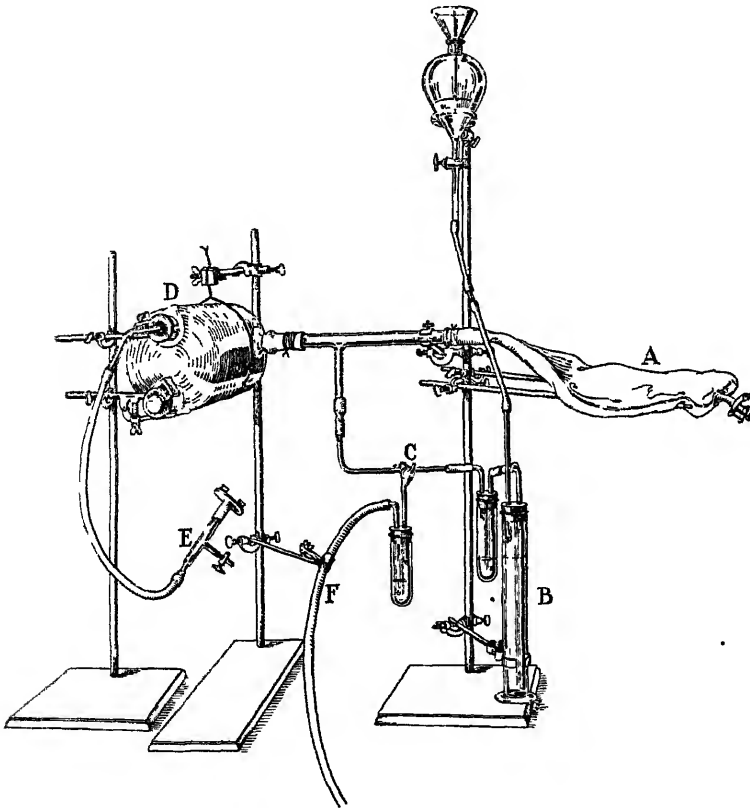
Fig 2 A, rubber bag, B, volumetric measuring cylinder for carbon monoxide, C, three-way stop-cock, D, Wolff bottle for removing the carbon dioxide, E, mouthpiece, F, tubing connected with the oxygen cylinder

18.5 volumes per cent of O_2 capacity, the capacity in this case would be 22.2 cc per 100 cc. of blood; per 3,700 cc. it accordingly would be 821.4 cc. One, therefore, could safely give 164 cc. of carbon monoxide reduced to standard conditions (760 mm. and 0°).

The blood sample was drawn without stasis from the arm vein

and kept under paraffin oil.   The carbon monoxide was determined·by the method of Van Slyke and Salvesen,[z] 3 cc. being used for each determination, as the carbon monoxide content of the blood is rather small in these experiments.

The air in the bag was analyzed for oxygen, carbon dioxide, and carbon monoxide after each experiment, as in the animal experiments.   In all except the last experiment the amount of carbon monoxide left was about 2 cc · 1.47, 1 902, 2.53, 1.832, 1.96; average 1.938 cc   In the last experiment it was 4.42 cc., but in this case more oxygen was given than necessary, and the oxygen content of the bag was 33 per cent.   This probably accounts for the slower absorption of the carbon monoxide, the dissociation curve of the carbon monoxide-hemoglobin being depressed when the oxygen percentage increases.   If the experiments, therefore, are always performed in the same way, the amount of carbon monoxide left is constant, and if the apparatus has the same capacity as used in the present experiments, the correction which must be subtracted is 2 cc.   The experiments done in this way are much simpler, the only determination which must be done being that of the carbon monoxide in the blood.

Six healthy individuals were examined, ranging in age from 23 to 37 years.   The material was rather uniform as all were young people without any adipositas.   The results are given in Table VI.

The average blood volume found was 3,888 cc., constituting $\frac{1}{16\ 8}$ of the weight, or 5.95 cc. per 100 gm.   The extremes are $\frac{1}{14\ 3}$ and $\frac{1}{19\ 08}$.   The largest volumes were found in Nos. 1 and 6, both of whom are tall and slim, especially No. 6 who is abnormally thin, and weighs much less than would correspond with his height.   In No. 1 two determinations were made and the difference found is only 7 cc. of blood.

The mean value, then, is a little larger than that of Plesch, who found $\frac{1}{17\ 9}$ and smaller than that of Douglas, who found in two persons $\frac{1}{13\ 9}$ and $\frac{1}{12.5}$

[z] Van Slyke, D D , and Salvesen, H. A , *J Biol Chem.*, 1919, xl, 103

All the persons experimented on felt comfortable, and did not have any disagreeable sensations. The breathing was easy, and even a certain degree of dyspnea is not likely to interfere with the use of this method in pathological cases. Former investigators

TABLE VI

Blood Determinations in Human Beings

No	Date	Name	Age	Weight	Duration	Hemoglobin	Blood volume		
							cc	Ratio	Cc per 100 gm
	1919		*yrs*	*kg*	*min*	*per cent*	*cc*		
1	July 28	Dr H S	30	68 8	32	114	4,594	$\frac{1}{14\ 97}$	6 67
	Aug 1	"		68 8	35	114	4,601	$\frac{1}{14\ 82}$	6 74
2	July 29	Mr A S	26	60 9	28	124 9	3,464	$\frac{1}{17\ 6}$	5 68
3	" 29	Dr H A	36	66 4	23	118	3,479	$\frac{1}{19\ 08}$	5 23
4	" 30	Dr V S	37	72 7	27	113	3,877	$\frac{1}{18\ 7}$	5 35
5	" 31	Dr J T	28	61	23	114	3,429	$\frac{1}{17\ 7}$	5 65
6	Aug 1	Mr R T	23	62 7	22	116	4,380	$\frac{1}{14\ 3}$	6 99
Average				65 4			3,888	$\frac{1}{16.8}$	5 95

have used the carbon monoxide method in heart, kidney, and anemic cases without any difficulties.

It is hoped that the blood volume method with the easy technique for the carbon monoxide determination will be of more practical value than before

SUMMARY.

Determinations of the blood volume by the carbon monoxide method with the simple technique previously described for the blood analysis have been made in fourteen rabbits and six normal men Eleven determinations in seven male rabbits show an average blood volume of $\frac{1}{20\,21}$ of the body weight, or 4.95 cc per 100 gm Nine determinations in five non-pregnant female rabbits show an average of $\frac{1}{19.99}$, or 5.0 cc. per 100 gm.

In two pregnant rabbits the blood volume was largely increased, absolutely and relatively, about a week post partum it was restored to normal again.

The blood volume of rabbits may change from time to time, but the total oxygen capacity remains constant.

Seven determinations in six healthy men show an average of $\frac{1}{16.8}$ of the body weight, or 5.95 cc. per 100 gm.

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A NOTE ON THE DETERMINATION OF CATALASE IN BLOOD.*

By MEYER BODANSKY

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During the course of several hundred determinations of cata-lase in the blood of normal and pathological individuals accord-ing to the method employed by Burge,[1] the author observed a consistent variation of from 15 to 35 per cent in the volume of oxygen evolved, depending upon the hydrogen peroxide solution used. The hydrogen peroxide used in these determinations was prepared by diluting commercial 3 per cent hydrogen peroxide solution with an equal volume of distilled water

Method

0 2 cc. of the blood was placed in a small crucible which was introduced into a bottle containing 100 cc of approximately 1 5 per cent (4.6 volume per cent) hydrogen peroxide and a drop of caprylic alcohol   The bottle, which was kept at 22°C in a water bath, was shaken 120 double shakes per minute for 10 minutes and the oxygen evolved was conducted through rubber tubes into inverted burettes and measured   The volume of oxygen obtained varied between 275 and 425 cc with different bloods.

In a later paper Burge[2] states that he found it necessary to use the same make of hydrogen peroxide in all the determinations as the different makes gave different results   For his work he

purchased 200 liters of hydrogen peroxide which was kept in a container in a dark, cool place.

It has been found, in this laboratory, that different samples of the same make of hydrogen peroxide often gave results which differed by 15 to 35 per cent   Since all the samples contained the same preservative (acetanilide) in approximately the same amount (0.05 per cent), it was assumed that the discrepancies were due to the difference in the acidity of the various samples.

A series of experiments was performed to test this assumption. A set of H_2O_2 solutions, 5.8 volumes per cent, containing varying amounts of HCl or NaOH was prepared.   As alkaline solutions of hydrogen peroxide decompose on standing, the solutions for these experiments were freshly prepared.

2 cc. of blood containing a slight trace of potassium oxalate were diluted to 200 cc. with distilled water.   5 cc of this solution, equivalent to 0.05 cc of the blood, were placed in a small crucible and introduced into a bottle containing 20 cc. of hydrogen peroxide solution and one drop of caprylic alcohol.   The bottle, kept at 22°C. in a water bath, was shaken 120 double shakes per minute for 10 minutes.   The oxygen evolved was conducted through rubber tubes into inverted burettes and the volumes measured at intervals of $\frac{1}{2}$ minute.   All gas volumes were calculated to standard conditions; i.e., 0°C. and 760 mm. Hg. pressure.

The results are given in Charts 1 and 2.

A reference to the charts will show the effect of acid and alkali on the velocity of the reaction.   The activity of the enzyme catalase was completely inhibited when the hydrogen ion concentration was pH 1, and was slight in a concentration of pH 2. The velocity of the reaction increases as the acidity of the hydrogen peroxide is decreased and is greatest when the solution is slightly alkaline.   The activity of the enzyme was considerably retarded when the concentration of the NaOH was above pH 11.

Falk, McGuire, and Blount[3] point out that there is no well' defined hydrogen ion concentration for maximum action with oxidase, peroxidase, and catalase.   According to Sorensen,[4] the·

[3] Falk, K G , McGuire, G , and Blount, E , *J. Biol Chem* , 1919, xxxviii, 237

[4] Sorensen, S P L , *Biochem Z* , 1909, xxi, 288 ·

optimum hydrogen ion concentration for catalase depends on the duration of the reaction. Thus he finds pH 6.73 the optimum for 320 minutes, and pH 7.1 for 40 minutes. With blood catalase under the experimental conditions stated above, I find the optimum to be about pH 7.5 for 10 minutes, about pH 8 for 5 minutes, and about pH 10 for 2 minutes.
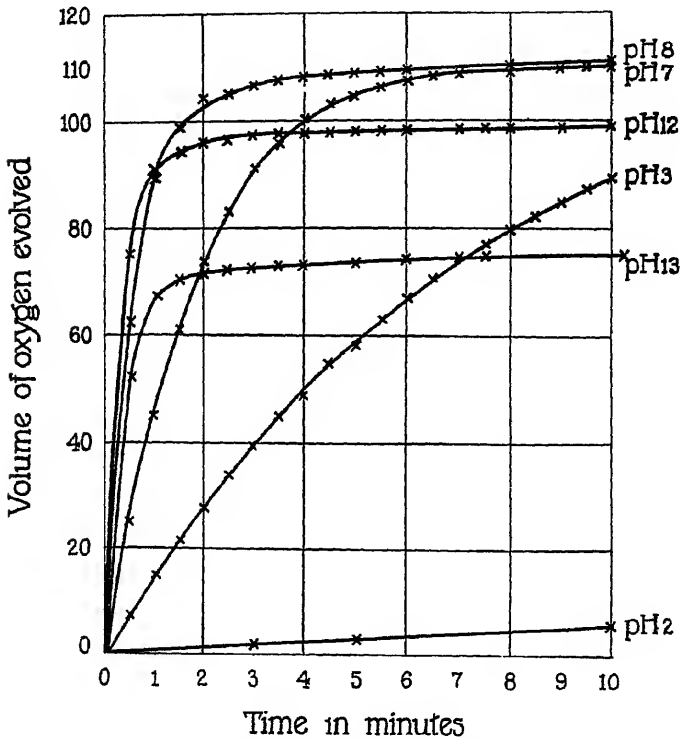
CHART 1 Curves showing the effect of the hydrogen ion concentration of the hydrogen peroxide on the velocity of the reaction between catalase and hydrogen peroxide

As far as I am aware, there is no satisfactory absolute method for the determination of catalase. Nearly all the methods in use give relative results Slight changes in temperature, impurities in, and the reaction of the hydrogen peroxide influence the

reaction very materially. These experiments emphasize the caution which must be exercised in maintaining uniform conditions especially when a series of determinations is made over a long period of time.

The author's thanks are due to Lieutenant T. E. Buckman and Lieutenant G. L. Foster of this laboratory for their helpful suggestions.

THE ACTION OF FURFUROL AND DEXTROSE ON AMINO-ACIDS AND PROTEIN HYDROLSATES.

By C T DOWELL and PAUL MENAUL

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While hydrolyzing the whole plant, including the seed, of some grain sorghums, we noticed that a large amount of furfurol was given off, and since we obtained an unusually large per cent of the nitrogen in the humin it occurred to us that this might be due to a reaction between the amino-acids and furfurol   A survey of the literature seems to confirm this idea   For example Grindley and Slater[1] found 15.79 per cent of the nitrogen in the humin formed in the hydrolysis of alfalfa hay, which was a much larger amount than that found in any other substance.   Nollau[2] obtained 13 75 per cent of humin nitrogen from wheat bran.   Hart and Sure[3] found 11.83 per cent of humin nitrogen when xylan was added in the hydrolysis of casein.   Gortner[4] studied the action of formaldehyde, benzaldehyde, and furfurol on certain amino-acids and states that the humin nitrogen is probably due in great part to the formation of furfurol in the hydrolysis of the protein

We found, in attempting to apply the Van Slyke method to analysis of the hydrolsate from the grain sorghum plant, not only a large amount of humin nitrogen but also incomplete precipitation of basic acids which was unexpected as 15 gm. of phosphotungstic acid had been added.   The addition of 50 gm. of the acid was required for complete precipitations   This amount precipitated from 30 to 33 per cent of the total nitrogen.   Only

[1] Grindley H S , and Slater, M E , J Am Chem. Soc., 1915, xxxvii, 2762

[2] Nollau, E H , J Biol Chem , 1915, xxi, 611

[3] Hart, E B , and Sure, B , J Biol Chem , 1916–17, xxviii, 241

[4] Gortner, R A , J Biol Chem , 1916, xxvi, 177.

a small amount of the precipitate redissolved on warming, nor was it possible to decompose it except to a slight extent with ether-amyl-alcohol mixture. Grindley, Nollau, and others who have applied this method to feedstuffs do not mention having had such difficulties. However, Brewster and Alsberg[5] in an article which appeared after we had decided to discontinue our efforts to apply the method state that they obtained a much larger per cent of nitrogen in the phosphotungstic precipitate than should be obtained, and attributed this partly to the precipitation of the humin nitrogen by the phosphotungstic acid. They had the same difficulty as we in dissolving the precipitate. It seemed to us that this trouble was caused by the humin which remained in solution even after it was made alkaline, but was precipitated by the phosphotungstic acid.

These difficulties led us to study the action of furfurol and dextrose on amino-acids and protein hydrolsates. One would expect that some of the amino-acids would react more readily with aldehydes and sugars than would others, and if this is true it would make the application of the Van Slyke method to feedstuffs useless. The results obtained by McHargue[6] in hydrolyzing casein in the presence of starch for different lengths of time might be due to the difference in reactivity of the amino-acids with sugars or aldehydes   We have succeeded in obtaining but a few of the amino-acids. However, it is thought worth while to report the results already obtained and also our study of the action of furfurol and dextrose on amino-acids and protein hydrolsates.

<div align="center">EXPERIMENTAL.</div>

<div align="center">*Action of Furfurol on Amino-Acids*</div>

Determination of the amino nitrogen was made by the Van Slyke method on the stock solution of the amino-acids and on the solution boiled with HCl and furfurol in acid solution. The results are shown in Table I. Two trials were made with glycine without getting evidence of a reaction between it and furfurol.

It was found that furfurol, when boiled with HCl, changes into a black humin-like mass and it was thought that the decrease in

[5] Brewster, J F , and Alsberg, C L , *J Biol Chem* , 1919, xxxvii, 367.
[6] McHargue, J S , *J. Agric Research*, 1918, xii, 1

TABLE I.

Action of Furfurol on Amino-Acids in 10 Per Cent HCl

Acid	Time boiled	Amount of furfurol	Volume of amino N_2 in 10 cc of solution	N_2 recovered
	hrs	*cc*		*per cent*
Tyrosine	2	0	5 9	89 8
"	2	1	5 3	
"	2	0	27 0	
"	2	2	24 5	90 7
Cystine	2	0	32 5	
"	2	1	27 5	84 6
Glycine	2	0	33 7	100 0
"	2	1 5	33 5	

amino nitrogen might be due to adsorption by this substance. To determine whether or not this was true 50 gm. of grain sorghum plant, ground to pass through a 20 mesh, were hydrolyzed in an autoclave at 20 pounds pressure in 10 per cent HCl for 3 hours (we had shown this to give complete hydrolysis) and the humin was washed with 2,500 cc. of hot water by decantation. It was then washed in the same way with another 2,500 cc. and this last was evaporated to 25 cc. and the amino nitrogen determined, 2.14 mg were obtained. The 18 gm. of humin contained 165.4'mg. of amino nitrogen. This with a similar result showed that our results with the amino-acids given in Table I were not due to adsorption.

Action of Furfurol on Protein Hydrolsates.

Samples of shrimp, casein, wool, and salmon were hydrolyzed in an autoclave as already described and, after filtering off the humin, portions of the solutions, which had been made neutral, were boiled in HCl solution with the addition of furfurol The results are shown in Table II. The substances were selected so as to have hydrolsates containing largely basic acids in some solutions and mono-acids in others.

TABLE II

Action of Furfurol on Protein Hydrolsates in Acid

Hydrolsate	Volume of amino N₂ in 10 cc of solution before treatment with furfurol	Amount of furfurol	Concentration of acid	Time of boiling	Volume of amino N₂ in 10 cc of solution after treatment with furfurol	Amino N₂ recovered
	cc	cc	per cent	hrs	cc	per cent
Shrimp	29 4	1	10	2	29 0	98 6
Casein	12.0	1	10	2	11 5	95 8
Wool	27 3	1	10	2	26 3	96 4
Salmon	39 0	1	10	2	36 3	93 0
Shrimp	16.4	2	5	16	13 1	79 8
Wool	17 4	2	5	16	13 5	77 5
Salmon	23 35	2	5	16	17 5	74 9

TABLE III.

Effect of Concentration of Acid on Reaction between Furfurol and Casein Hydrolsate

Concentration of HCl	Time of boiling	Amount of furfurol	Volume of amino N₂ in 10 cc of solution before boiling	Volume of amino N₂ after boiling	Amino N₂ recovered
per cent	hrs	cc			per cent
0	15	2	33 4	17 6	51 1
0	15	2	31 1	21 3	68 4
5	15	2	30 2	26 3	87 0
10	15	2	30 2	27 3	90 3
20	15	2	16 3	15 7	96 3

TABLE IV

Effect of Concentration of Acid on Reaction between Dextrose and Casein Hydrolsates

Concentration of HCl	Time of boiling	Concentration of Dextrose	Volume of amino N₂ in 10 cc of solution before boiling	Volume of amino N₂ after boiling	Amino N₂ recovered
per cent	hrs	per cent			per cent
20	15	4	33 4	33 4	100 0
20	15	4	31 1	31 1	100 0
20	15	4	16 3	16 0	98 1
10	15	4	30 2	28 9	95 3
5	15	4	30 2	28 8	95 3
Neutral	15	4	33 4	28 1	84 1
"	15	4	31 1	26 0	83 6

TABLE V.

Effect of Boiling Casein Hydrolsate in 20 Per Cent HCl for 15 Hours in the Presence of Arabinose, Starch, Gum Arabic, and Cellulose.

Name of substance	Amount of substance	Volume of amino N_2 in 10 cc of solution before boiling	Volume of amino N_2 after boiling	Amino N_2 recovered
	gm			*per cent*
Arabinose	1	16 3	15 4	94 4
Gum Arabic	2	16 3	15 9	97 5
Starch	2	16 3	15 8	96 9
Cellulose	2	16 3	15 8	96 9

SUMMARY.

1 Glycine does not react with furfurol. There is no explanation of this since tyrosine and cystine give decided evidence of reaction

2. It is shown that but a slight amount of the humin nitrogen is due to adsorption.

3. The hydrolsates of shrimp, casein, wool, and salmon react with furfurol.

4 The greatest decrease in amino N_2 is obtained when the reaction between a protein hydrolsate and furfurol takes place in a neutral or slightly acid solution. This is probably due to the fact that furfurol forms a humin-like mass wh boiled in acid solution.

5. The greatest effect is obtained in the reaction between dextrose and a protein hydrolsate when the solution is either neutral or slightly acid. This shows that dextrose reacts directly with the amino-acids and not through the intermediate formation of furfurol as we thought might be true.

Our results with dextrose together with the fact that we seemed to get a precipitation of humin nitrogen with phosphotungstic acid leads us to think that the method proposed recently by Eckstein and Grindley[7] for the analysis of feeds⁺ ⁻s by the Van Slyke method would lead to low results. In order to remove the carbohydrates, they digest the sample for 60 hours in 0.1 per cent

[7] Eckstein, H. C , and Grindley, H S , *J. Biol Chem*, 1919, xxxvii, 373.

hydrochloric acid. This converts starches to sugars, and since the acid concentration is low, the conditions are most favorable for the reaction of the sugars with the amino-acids. It is true that this concentration of acid would not hydrolyze much of the protein, but it should be remembered that there is a high per cent (30 to 40 per cent) of soluble nitrogen, mostly in the form of amino-acids, in many feedstuffs

6 The results given in Table V show, as would be expected, that substances which yield either sugars or furfurol when boiled with hydrochloric acid decrease the amino nitrogen when boiled with a protein hydrolsate.

7. It was found that complete hydrolysis of various proteins is brought about by heating in 10 per cent HCl in an autoclave for 3 hours at a pressure of 20 pounds.

THE HEAT COAGULATION OF MILK.[*]

By H. H. SOMMER AND E. B. HART.

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison.)

The coagulation of milk by heat was first observed by Hammersten,[1] who found that it occurred at from 130–150°C., with different samples of milk. Since then the question has been studied very little, and no tenable explanation has ever been given for the difference in the coagulating points of milks from different cows. In recent years a knowledge of the factors which determine this difference has become very desirable, for these same factors undoubtedly determine whether a condensed milk will coagulate when it is sterilized. The coagulation of condensed milk on sterilizing causes serious losses in the milk-condensing industry.

In the manufacture of condensed milk, the fresh milk is first pasteurized or "preheated" at from 180–210°F. for from 1 to 20 minutes. The condensing is done under vacuum at 130–160°F. After the desired concentration has been attained, the milk is put into cans, sealed, and then sterilized at 224–240°F. for 20 to 50 minutes. It is during the sterilizing process that the coagulation occurs. Because it occurs so frequently, all the condensed milk is placed into shaking machines to break up any loose coagulum that may have formed. However, frequently the coagulum is so firm that even after shaking the milk remains lumpy. Such a product is rejected by the consuming public, and thus is a loss.

Manufacturers have sought to solve the problem by controlling the acidity of the milk. They have set an arbitrary standard such as 0.18 per cent acid (calculated as lactic acid), above which

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[*] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Quoted from literature referred to in, Kastle, J. H., Chemistry of milk, *Hyg. Lab. Bull. 56*, 1909.

they reject all milk   This has led to much difficulty, because often, immediately after it is drawn from the cow, milk has a higher titratable acidity than 0.18 per cent   Thus the condenseries may be rejecting perfectly fresh milk, believing that they are remedying their difficulty in this way, although it has never been demonstrated that titratable acidity is related to the coagulation.   The factors involved in the coagulation have never been determined, and no explanation is available on which to base a remedy for this difficulty

To offer an explanation for the difference in the coagulating points of different milk samples the following factors were studied: titratable acidity, hydrogen ion concentration, concentration of the milk, and composition and balance of the milk salts.

The Heat Test.

The temperature at which the milk was heated was arbitrarily set at 136°C.   At first the heating was done in an autoclave at 50 pounds pressure for 20 minutes, and in that way the milks were differentiated into coagulating and non-coagulating.   With the autoclave, it took about 10 minutes to get up to the desired pressure; and, after the milk had been heated, the pressure had to be released gradually to prevent the milk from boiling over.   The disadvantages of this method were such that there were no sharp limits from which to calculate the 20 minute interval, and it was impossible to determine the relative rates at which the milk samples coagulated.

To overcome these disadvantages the·milk was placed into· small glass tubes, sealed, and then heated in a xylene vapor bath which was constant at 136°C. within 0 5°C   The sealed tubes were clamped in a rack, so arranged that it could be tilted to invert the tubes, to see how the milk would flow, and in that way it was possible to determine the exact length of time required for each sample to coagulate   The milk in the sealed tubes was up to 136°C. in less than 1 minute, so the point from which to calculate the time was practically the instant the tubes were inserted into· the vapor bath.

Titratable Acidity.

Since condensenes are attempting to remedy the coagulation problem by rejecting milk above 0.18 per cent acid (calculated as

TABLE I

Titratable Acidity and Coagulation.

May 8, 1919			May 10, 1919			May 16, 1919		
Cow No	Titratable acidity	Coagulation	Cow No	Titratable acidity	Coagulation	Cow No	Titratable acidity	Coagulation
	Lactic acid			Lactic acid			Lactic acid	
	per cent	min		per cent	min		per cent	min
1	0 257	20—*	1	0 241	6	31	0 203	4½
2	0 235	3	2	0 231	11	2	0 193	20—
3	0 216	20—	26	0 228	5	1	0 192	20—
4	0 214	20—	31	0 222	5½	3	0 188	4½
5	0 210	8	27	0 212	6	27	0 188	6½
6	0 207	20—	3	0 212	5½	6	0 188	20—
7	0 206	6	4	0 211	20—	4	0 188	20—
28	0 201	20—	5	0 205	5½	28	0 186	20—
9	0 200	20—	9	0 197	20—	12	0 184	10
10	0 200	20—	28	0 199	20—	26	0 184	3
11	0 195	4½	6	0 195	20—	5	0 183	6½
12	0 191	20—	29	0 192	5½	9	0 182	20—
13	0 190	20—	10	0 190	20—	14	0 182	9
14	0 189	6½	13	0 186	9	29	0 182	1¾
15	0 182	20—	12	0 185	11	11	0 178	3½
16	0 179	4	11	0 184	5	13	0 175	4
8	0 174	9	8	0 174	20—	10	0 171	20—
17	0 172	20—	14	0 175	6½	30	0 165	20—
18	0 167	20—	15	0 172	20—	15	0 163	20—
19	0 158	12	17	0 166	20—	8	0 163	6½
20	0 157	20—	18	0 160	20—	17	0 156	20—
21	0 156	20—	30	0 162	4	18	0 154	20—
22	0 148	3	7	0 162	2	7	0 148	2
23	0 146	3½	21	0 157	20—	20	0 143	20—
24	0 143	20—	20	0 147	20—	19	0 135	20—
25	0 120	2	23	0 145	5½	22	0 133	2
			19	0 144	20—	23	0 130	4
			22	0 144	2	24	0 128	20—
			24	0 141	20	21	0 128	20—
			25	0 131	1½	25	0 102	1½

*20— = no coagulation in 20 minutes

lactic acid), it was of interest to know how much variation there
was in the titratable acidity of milk from individual cows, and
what relation the acidity would bear to the coagulation.   To study
this, samples were taken from the University herd and titrated
immediately, and the heat test in the xylene vapor bath applied
as soon as possible.   The results given in Table I were obtained.

The titratable acidity varies from 0.102 to 0 257 per cent.   Out
of the 86 samples, 45 are above 0.18 per cent.

In fresh milk there is no direct relation between titratable
acidity and coagulation, as is evident from Table II.   If fresh
milk samples were more nearly alike in titratable acidity, then
titratable acidity might bear a direct relationship to the heat

TABLE II

Summary of Titratable Acidity and Coagulation

Date	No of samples	No above 0 18 per cent acid *	No that are 20 + †	No below 0 18 per cent acid ‡	No that are 20 +
May 8	26	15	5	11	6
" 10	30	14	7	16	7
" 16	30	16	11	14	6
Total .	86	45	23	41	19

* Per cent above 0 18 per cent acidity, coagulating 20+ = 51 2 per cent
† 20+ means coagulation within 20 minutes
‡ Per cent below 0 18 per cent acidity, coagulating 20+ = 46 4 per
cent

coagulation of commercial milk samples.   The acidity would then
be a measure of the amount of fermentation that had taken place
Lactic acid fermentation lowers the coagulating point in two ways,
(1) it changes the reaction, and (2) it lowers the citric acid content
of the milk very rapidly.[2]   Both of these are factors in lowering
the coagulating point, as will be shown later.

Since fresh milk samples vary so widely in titratable acidity,
it is impossible to measure the extent of acid fermentation in a
sample by titration.   For this reason it is impossible to use titra-
table acidity as a criterion of coagulability.

[2] Bosworth, A. W , and Prucha, M J , *Tech. Bull. 14, N. Y. Agric Exp
Station*, 1910.

TABLE III

Hydrogen Ion Concentration and Coagulation

Date	Cow	pH	C_H	Coagulation in 20 min
1919				
Feb 21	31	6 55	$2 82 \times 10^{-7}$	+++*
	32	6 83	$1 48 \times 10^{-7}$	+
	13	6 58	$2 63 \times 10^{-7}$	+++
	24	6 83	$1 48 \times 10^{-7}$	—
" 25	13	6 25	$5 62 \times 10^{-7}$	—
	32	6 66	$2 19 \times 10^{-7}$	—
	31	6 66	$2 19 \times 10^{-7}$	+++
" 26	13	6 58	$2 63 \times 10^{-7}$	+++
	32	6 69	$2 04 \times 10^{-7}$	++
	31	6 70	$1 99 \times 10^{-7}$	++
	24	6 73	$1 86 \times 10^{-7}$	+
	14	6 70	$1 99 \times 10^{-7}$	+++
" 27	13	6 44	$3 62 \times 10^{-7}$	+
	32	6 64	$2 29 \times 10^{-7}$	++
	31	6 64	$2 29 \times 10^{-7}$	+++
" 27	24	6 70	$1 99 \times 10^{-7}$	—
	14	6 44	$3 62 \times 10^{-7}$	—
	33	6 59	$2 56 \times 10^{-7}$	+++
" 28	13	6 50	$3 16 \times 10^{-7}$	—
	31	6 94	$1 15 \times 10^{-7}$	+++
	32	6 92	$1 20 \times 10^{-7}$	—
	24	6 93	$1 17 \times 10^{-}$	++
	14	6 50	$3 16 \times 10^{-7}$	—
	33	6 68	$2 09 \times 10^{-7}$	+++
Mar 3	13	6 67	$2 14 \times 10^{-7}$	—
	31	6 84	$1 44 \times 10^{-7}$	—
	33	6 69	$2 04 \times 10^{-7}$	++
" 5	33	6 64	$2 29 \times 10^{-7}$	+++
	13	6 64	$2 29 \times 10^{-7}$	—
	31	6 93	$1 17 \times 10^{-7}$	+
	32	6 79	$1 62 \times 10^{-7}$	—
	24	6 79	$1 62 \times 10^{-7}$	—
	14	6 58	$2 63 \times 10^{-7}$	—
" 6	24	6 97	$1 07 \times 10^{-7}$	+
	33	6 59	$2 57 \times 10^{-7}$	+++
" 7	31	6 85	$1 41 \times 10^{-7}$	+++
" 10	33	6 79	$1 62 \times 10^{-7}$	+++

* Number of plus signs indicates degree of firmness.

Hydrogen Ion Concentration.

Titratable acidity does not give an index to true acidity, or hydrogen ion concentration, so that if there is any relation between acidity and·coagulation, it would be most likely to exist between the hydrogen ion concentration and coagulation. To study this possibility the hydrogen ion concentration of fresh milk was determined by means of the gas chain method, and the heat test was applied by means of the autoclave. The results given in Table III were obtained.

From a study of the data it becomes evident that the hydrogen ion concentration is not the determining factor in the coagulation. Samples of equal C_H do not always respond alike to the heat test, one may remain liquid, and the other may form a firm coagulum. In a large number of cases samples of high C_H did not coagulate, whereas samples of lower C_H did, the exact reverse of what should happen if true acidity was the cause of the coagulation.

We must conclude from this that in fresh milk C_H is not the determining factor in the coagulation. However, it may become a factor, for if we change the reaction of a milk sample by adding small amounts of acids the coagulating point is lowered.

Concentration.

The concentration of the milk would be expected to influence the coagulating point. This was found to be the case when milk was diluted (Table IV).

TABLE IV

Relation of Coagulation to Concentration

25 cc of milk + H_2O	Coagulation time
H_2O added	
cc	min
0 0	$1\frac{1}{2}$
1 0	2
2 0	$2\frac{1}{4}$
3 0	14
4 0	35—
5.0	35—
6 0	35—

Not only the concentration of the casein influences the coagulating point, but also the concentration of the serum. This was determined by comparing the effect of water dilution to the effect of dilution with milk serum obtained by filtering the milk through Pasteur-Chamberlain filters (Table V).

In the dilution with water, where the casein and the serum are both diluted, the effect is greater than where the casein alone is diluted by adding serum; therefore, the concentration of the serum is also a factor influencing the coagulating point.

Concentration of casein and of serum may in part explain the difference in the coagulating points of different milk samples.

TABLE V

Relation of Coagulation to Concentration of Serum

25 cc of milk + serum	Coagulation time
Serum added	
cc	min
0 0	$1\frac{1}{2}$
0 1	$1\frac{3}{4}$
0 2	2
25 cc of milk + H_2O	Coagulation time
H_2O added	
cc	min
0 0	$1\frac{1}{2}$
0 1	$2\frac{1}{2}$
0 2	4

However, in most cases, with the slight variation in concentration, this factor is of minor importance, just as C_H is. There must be another factor of greater importance.

Composition and Balance of Milk Salts

Since electrolytes have a very marked effect upon the stability of colloids, we should expect that variations in the salt composition would influence the stability of the casein in the milk

That the various salts exert an influence on the coagulating point was shown in a number of cases

The effect of an addition of ammonium oxalate to milk that previously coagulated is shown in Table VI.

The removal of calcium by precipitation prevents coagulation in most cases and similarly in most cases the addition of small amounts of calcium salts lowers the coagulating point. This coagulation can again be balanced by means of sodium citrate or dipotassium phosphate (Tables VII, VIII, IX, and X). Coagulation caused by $MgCl_2$ or $BaCl_2$ can also be balanced by sodium citrate (Tables XI and XII).

In most cases coagulation can be prevented by the addition of citrates or phosphates, the coagulation being due to an excess of calcium and magnesium. However, in a few cases the addition of citrates or phosphates did not prevent coagulation, but rather

TABLE VI.

Ammonium Oxalate Prevents Coagulation.

5 cc of milk + 10 per cent $(NH_4)_2 C_2O_4$	Coagulation in 20 min
$(NH_4)_2 C_2O_4$ added	
drops	
0	+++
1	++
2	+
3	−
4	−−

hastened it. In these cases the addition of the proper amount of calcium salts prevents coagulation or at least raises the coagulating point (Tables XIII and XIV).

From the data we see that the calcium and magnesium are balanced by the phosphates and citrates of the milk practically in gram-equivalent amounts. The sodium and potassium chlorides in the concentrations present do not have any marked influence on the coagulating point, so that the balance of the four constituents, calcium, magnesium, citrates, and phosphates, largely determines whether a milk will coagulate or not. If calcium and magnesium are in excess, the milk will coagulate on heating. If calcium and magnesium are properly balanced with the phosphates and citrates, the optimum stability obtains. If phosphates and citrates are in excess, coagulation will also result.

TABLE VII.

Balance between Calcium and Citrates *

25 cc of milk plus			Coagulation time
M/2 Ca acetate	M/2 Na citrate	H₂O	
cc	cc	cc	min
0 0	0 0	1 3	3
0 3	0 0	1 0	$\frac{1}{2}$
0 3	0 1	0 9	$2\frac{1}{2}$
0 3	0 2	0 8	.3
0 3	0.3	0 7	2
0 3	0 4	0 6	$1\frac{1}{2}$

*The sodium citrate consisted of 25 cc of sodium M/2 citrate plus 3 cc of M/2 citric acid. This solution was distinctly acid, so that the balancing effect could not have been due to neutralization of acidity by means of the sodium citrate.

TABLE VIII

Balance between Calcium and Citrates *

25 cc of milk plus			Coagulation time.
M/2 Ca acetate	M/2 Na citrate	H₂O	
cc	cc	cc	min
0 0	0 0	1 6	4
0 4	0 0	1 2	$\frac{1}{2}$
0 4	0 2	1 0	40—
0 4	0 4	0 8	40—
0 4	0 6	0 6	$2\frac{1}{4}$
0 4	0 8	0 4	2

*The sodium citrate consisted of 25 cc of M/2 sodium citrate plus 1 cc of M/2 citric acid

TABLE IX

Balance between Calcium and Citrates

25 cc of milk plus			Coagulation time
M/2 Ca acetate	M/2 Na citrate	H₂O	
cc	cc	cc	min
0 0	0 0	1 8	25—
0 8	0 0	1 0	$\frac{1}{8}$
0 8	0 4	0 6	$\frac{1}{8}$
0 8	0 6	0 4	25—
0 8	0 8	0 2	25—
0 8	1 0	0 0	$4\frac{1}{2}$

TABLE X

Balance between Calcium and Phosphates

25 cc of milk plus			Coagulation time
M/2 Ca acetate	M/2 K_2HPO_4	H_2O	
cc	cc	cc	min
0 0	0 0	1 2	20 —
0 5	0 0	0 7	$\frac{1}{4}$
0 5	0 2	0 5	$\frac{3}{8}$
0 5	0 3	0 4	1 .
0 5	0 4	0 3	20 —
0 5	0 5	0 2	20 —
0 5	0 6	0 1	20 —
0 5	0 7	0 0	6

TABLE XI

Balance between Magnesium and Citrates

25 cc of milk plus		•	Coagulation time
M/2 $MgCl_2$	M/2 Na citrate	H_2O	
cc	cc.	cc	min
0 0	0 0	0 7	20 —
0 J	0.0	0 4	$\frac{1}{2}$
0 3	0 2	0 2	20 —
0 3	0.3	0 1	20 —
0 3	0 4	0 0	S

TABLE XII

Balance between Barium and Citrates

25 cc of milk plus			Coagulation time
M/2 $BaCl_2$	M/2 Na citrate	H_2O	
cc	cc	cc	min .
0 0	0 0	0 4	20 —
0 2	0 0	0 2	$\frac{1}{6}$
0 2	0 2	0 0	20 —

Thus the coagulation of a milk sample on heating may be due either to an excess or a deficiency of calcium and magnesium We may explain this in the following manner. The casein of the milk is most stable with regard to heat coagulation when it is in combination with a definite amount of calcium   If the calcium

combined with the casein is above or below this optimum, the casein is not in its most stable condition.   The calcium in the milk distributes itself between the casein, citrates, and phosphates chiefly.   If the milk is high in citrate and phosphate content, more calcium is necessary in order that the casein may retain its optimum calcium content after competing with the citrates and phosphates.   If the milk is high in calcium, there may not be

TABLE XIII.

A Sample in Which Calcium Prevents Coagulation

25 cc of milk plus			Coagulation time
M/2 Ca acetate	M/2 Na citrate	H_2O	
cc	cc	cc	min
0 0	0 0	0 8	$1\frac{1}{2}$
0 2	0 0	0 6	20 —
0 2	0 1	0 5	$1\frac{1}{4}$
0 2	0 2	0 4	1
0 2	0 3	0 3	$\frac{3}{4}$
0 2	0 4	0 2	$\frac{3}{4}$

TABLE XIV

A Sample in Which Calcium Raises the Coagulating Point

25 cc of milk plus		Coagulation time
M/4 Ca acetate	H_2O	
cc	cc	min
0 0	0 5	$1\frac{3}{4}$
0 1	0 4	2
0 2	0 3	$2\frac{1}{4}$
0 3	0 2	3
0 4	0 1	6
0 5	0 0	$2\frac{1}{2}$

sufficient citrate and phosphate to compete with the casein to lower its calcium content to the optimum.   In such a case the addition of citrates or phosphates makes the casein more stable by reducing its calcium content.   The magnesium functions by replacing the calcium in the citrates and phosphates.

In most cases the coagulation is due to an excess of calcium and magnesium   It is possible to balance this excess by citrates,

TABLE XV

Analyses to Show Balance between Salts as Related to Coagulation

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Sample No	Citric acid	P$_2$O$_5$	CaO	MgO	Citric acid Gram-equivalent	P$_2$O$_5$ Gram-equivalent	CaO Gram-equivalent	MgO Gram-equivalent	Citric acid +P$_2$O$_5$ Gram-equivalent	CaO + MgO Gram-equivalent	Column 11 minus Column 10	Column 12 minus 0 40	Coagulation time	Total solids
	per cent	per cent	per cent	per cent									min	per cent
1	0 100	0 216	0 189	0 023	0 52	1 94	3 38	0 56	2 46	3 94	+1 48	+1 08	1⅔	12 41
2	0 292	0 255	0 194	0 021	1 52	2 30	3 41	0 51	3 82	3 98	+0 16	−0 24	1¾	15 02
3	0 191	0 236	0 184	0 025	0 99	2 13	3 29	0 62	3 12	3 91	+0 79	+0 39	2	13 55
4	0 130	0 193	0 148	0 022	0 68	1 74	2 64	0 55	2 42	3 19	+0 77	+0 37	2	9 79
5	0 165	0 292	0 189	0 022	0 86	2 63	3 38	0 55	3 49	3 93	+0 44	+0 04	3	15 20
6	0 145	0 277	0 210	0 021	0 76	2 50	3 75	0 53	3 26	4 28	+1 02	+0 62	3½	15 25
7	0 220	0 237	0 185	0 028	1 15	2 13	3 30	0 70	3 28	4 00	+0 72	+0 32	4	14 40
8	0 240	0 200	0 179	0 024	1 25	1 80	3 19	0 60	3 05	3 79	+0 74	+0 34	4	13 19
9	0 220	0 294	0.181	0 029	1 15	2 65	3 23	0 74	3 80	3 97	+0 17	−0 23	4½	14 30
10	0 207	0 280	0 204	0 025	1 08	2 52	3 64	0 63	3 60	4 27	+0 67	+0 27	4½	14 76
11	0 213	0 287	0 196	0 027	1 11	2 58	3 50	0 67	3 69	4 17	+0 48	+0 08	6½	14 14
12	0 164	0 278	0 190	0 026	0 85	2 49	3 39	0 65	3 34	4 04	+0 70	+0 30	6½	14 98
13	0 163	0 233	0 184	0 026	0 85	2 10	3 28	0 64	2 95	3 92	+0 87	+0 47	6½	14 02
14	0 124	0 258	0 170	0 025	0 65	2 32	3 04	0 62	2 97	3 66	+0 69	+0 29	9	14 14
15	0 312	0 246	0 204	0 023	1 62	2 21	3 64	0 59	3 83	4 24	+0 41	+0 01	10	13 23
16	0 194	0 273	0 158	0 024	1 01	2 46	2 82	0 60	3 47	3 42	−0 05	−0 45	20—	13 34
17	0 185	0 252	0 167	0 021	0 96	2 25	2 98	0 52	3 21	3 50	+0 29	−0 11	20—	13 75
18	0 168	0 262	0 164	0 026	0 88	2 36	2 93	0 65	3 24	3 58	+0 34	−0 06	20—	12 47

19	0 192	0 234	0 160	0 021	1 00	2 11	2 86	0 53	3 11	3 39	+0 28	−0 12	20 —	12 61
20	0 210	0 249	0 193	0 025	1 09	2 24	3 45	0 61	3 33	4 06	+0 07	+0 27	20 —	12 65
21	0 234	0 240	0 132	0 026	1 22	2 16	2 36	0 64	3 38	3 00	−0 38	−0 78	20 —	14 17
22	0 213	0 196	0 145	0 019	1 11	1 76	2 50	0 46	2 87	3 05	+0 18	−0 22	20 —	10 81
23	0 237	0 253	0 176	0 021	1 23	2 28	3 14	0 52	3 51	3 66	+0 15	−0 25	20 —	13 41
24	0 225	0 240	0 158	0 022	1 17	2 16	2 82	0 55	3 33	3 37	+0 04	−0 36	20 —	14 09
25	0 170	0 238	0 148	0 025	0 89	2 14	2 64	0 62	3 03	3 26	+0 33	+0 07	20 —	14 39
26	0 243	0 196	0 164	0 023	1 27	1 77	2 93	0 57	3 04	3 50	+0 46	+0 06	20 —	11 34
27	0 216	0 219	0 156	0 022	1 13	1 97	2 79	0 55	3 10	3 34	+0 24	−0 16	20 —	12 59
28	0 238	0 291	0 162	0 022	1 24	2 62	2 89	0 54	3 86	3 43	−0 43	−0 83	20 —	12 67
29	0 161	0 224	0 179	0 020	0 84	2 02	3 19	0 49	2 86	3 68	+0 92	+0 42	20 —	13 60
30	0 144	0 206	0 139	0 020	0 75	1 85	2 48	0 51	2 60	2 99	+0 39	−0 01	20 —	11 36

phosphates, carbonates, and other salts.   It is also stated that
danger of coagulation may be avoided in the actual practice of
condensing milk by lengthening the "preheating" period, using
higher temperatures.   This may have the effect of lowering the
soluble calcium content by precipitating part of it as insoluble
calcium phosphate [1]

To demonstrate the importance of the salt balance in the coagu-
lation of milk, a number of samples were analyzed for total citric
acid, phosphorus, calcium, and magnesium (Columns 2, 3, 4, and
5, Table XV).   To calculate the balance between citric acid and
phosphates, and calcium and magnesium the percentages were
converted into gram-equivalents as follows·

$$(a) \quad \frac{\text{Citric acid}}{192} \times 100$$

$$(b) \quad \frac{P_2O_5 \times 100}{71} \times \frac{7}{11}*$$

$$(c) \quad \frac{CaO \times 100}{56}$$

$$(d) \quad \frac{MgO \times 100}{40}$$

* Multiply by $\frac{7}{11}$ because at pH 6 50, the average reaction of milk, the
ratio of primary to secondary phosphate, is such that the mean basicity
of the phosphates is approximately $\frac{7}{11}$ of what it would be if all the
phosphates were secondary phosphates

Column 10 shows the sum of citric and phosphoric acids in
gram-equivalents; Column 11, the sum of calcium and magnesium
in gram-equivalents   Column 12 shows the balance; a plus sign
showing an excess of calcium and magnesium, and a minus sign
showing an excess of citric and phosphoric acids.   In only a few
cases is there an excess of citric and phosphoric acids, and the
excess is small.   Those that coagulated had the largest excess of
calcium and magnesium.   To make this result more apparent,
Column 13 shows the values of Column 12 minus 0.40.   This figure
was arbitrarily chosen and subtracted so as to make the coagulat-
ing samples have a plus sign and the non-coagulating samples have
a minus sign.   In five cases out of the thirty this result does not
hold.   However, the fact that, in twenty-five out of the thirty
samples, those having the highest excess of calcium and magnesium

over citrates and phosphates coagulated and those having the lowest excess did not coagulate, indicates that this factor is very important.

The five exceptions may be due to the other factors, concentration and reaction. Samples 2 and 9, with their small excesses of calcium and magnesium, should not coagulate; however, both samples are high in total solids. Samples 20 and 26 did not coagulate although the excess of calcium and magnesium is high; again the explanation may lie partly in the concentration of the milk, both samples being low in total solids. If the pH had been determined we might have gained further insight into these exceptions and an explanation for the irregularity of Sample 29.

SUMMARY AND CONCLUSIONS.

1. The main factor in the heat coagulation of fresh milk is the composition of the milk salts. Apparently casein requires a definite optimum calcium content for its maximum stability. The calcium content of casein is largely controlled by the magnesium, citrates, and phosphates present.

2. In fresh milk there is no relation between titratable acidity and heat coagulation.

3. Acid fermentation in milk lowers the coagulating point by changing the reaction and by lowering the citric acid content. However, the titratable acidity of fresh milk samples varies so widely that it is impossible to determine the extent of acid fermentation by titration. Therefore it is impossible to use the acidity of milk as a criterion of coagulability.

4. Difference in concentration accounts partly for the difference in coagulation of fresh milk samples.

5. Hydrogen ion concentration is not the determining factor in fresh milk coagulation. It is nevertheless a factor in fresh milks, and in commercial milks it may become an important factor.

THE ACTION OF INTRAVENOUS INJECTIONS OF PANCREAS EMULSIONS IN EXPERIMENTAL DIABETES.

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It is evident that the demonstration of a beneficial effect of a pancreas preparation, when administered parenterally to a diabetic animal, would be of importance both theoretically and practically.   Theoretically it would support the internal secretion hypothesis of the origin of diabetes   Practically it would suggest a possible therapeutic application

The early work in this field was either negative or, as Pfluger,[1] and Leschke[2] have shown, is open to serious criticism   The usual criterion was a lowering of the concentration of sugar in the urine or a diminution of the total 24 hour output of sugar   Either of these effects may easily be caused by a diminished intake of food, due to the loss of appetite usually resulting from the treatment, to a change in the character of the diet, or to an influence on the kidneys   In clinical experience, the *feeding* of pancreas preparations has sometimes seemed to have a favorable subjective effect but with no constant antidiabetic action   Often this appeared to be due to supplying the *external* secretion to patients in need of it   Parenteral administration usually has not given good results   In a brief résumé of the literature Allen[3] says "Though pancreas feeding may have at least a digestive value in some cases of diabetes, injections of pancreatic preparations have proved both useless and harmful   The failure began with Minkowski, and has continued to the present without an exception "

The more recent work may be summarized very briefly   Scott[4] found that intravenous injections of water extracts of pancreatic tissue, which had previously been extracted with alcohol, diminished temporarily the

[1] Pfluger, E , *Arch ges Physiol* , 1907, cxviii, 267

[2] Leschke, E , *Arch Physiol* , 1910, 401

[3] Allen, F M , Studies concerning glycosuria and diabetes, Cambridge, 1913, 815

[4] Scott, E L , *Am J Physiol* , 1911–12, xxix, 306

sugar excretion of diabetic dogs and lowered their D/N ratio. Murlin and Kramer[5] injected boiled pancreatic extracts into depancreatized dogs and found a temporary reduction in the output of sugar. A mixed boiled extract of pancreas and duodenal mucosa produced a greater fall, and in one case a complete disappearance of the urinary sugar The results were referred by these authors mainly to the sodium carbonate present in the extracts, because Ringer's solution, which had been brought to about the same degree of alkalinity as the medium used for the extract, was found to have similar effects Later the preliminary announcement[6] of the present work led Murlin and Kramer[7] to try unboiled pancreatic extract. This was given by mouth, however, and was mixed with Na_2CO_3 A favorable influence on the respiratory quotient was observed in two experiments.

The present work was undertaken because of the remarkable influence which an emulsion of pancreas had been found to exert upon the disposition of intravenously injected dextrose in diabetic dogs In normal animals[8] large quantities of sugar, introduced intravenously, were promptly lost from the circulation, in diabetic animals,[6,9] on the other hand, this occurred very slowly, as was shown by the fact that even an hour and a half after the end of the sugar infusion the blood sugar was still far above its original level. Now, when an emulsion of pancreas was mixed with the glucose solution to be injected, the diabetic animal handled the sugar in nearly a normal manner.[6] Then the question naturally arose whether a similar pancreatic emulsion would not help the diabetic organism to dispose of its own excess sugar also.

In testing this question there were two principles which, we felt, should be followed. First, since the chemical properties of the effective substance or substances in the pancreas were unknown, it seemed necessary to avoid complicated procedures of purification; therefore, a simple water extraction with subsequent dilution with saline was adopted. Secondly, since depancreatization brings on its effect within a very short time, it appeared that normally the pancreas secretes its effective substance into the blood stream continually in very small amounts. It was therefore decided to

[5] Murlin, J R , and Kramer, B , *J. Biol. Chem* , 1913, xv, 365

[6] Kleiner, I S , and Meltzer, S. J , *Proc. Nat Acad Sc* , 1915, i, 338.

[7] Murlin, J R , and Kramer, B , *J Biol. Chem* , 1916, xxvii, 517.

[8] Kleiner, I S , and Meltzer, S J , *Am J Physiol* , 1914, xxxii, p. xvii Kleiner, I S , *J Exp Med* , 1916, xxiii, 507.

[9] Kleiner, I S , and Meltzer, S J , *Proc Soc. Exp. Biol and Med* , 1914–15, xii, 58

introduce the pancreatic preparation by the intravenous route very slowly and over an appreciable period, the results, however, might be expected to be observed during the injection and for only a few hours thereafter. These principles were followed and the results were very gratifying—in a word, the slow intravenous infusion of an aqueous pancreatic "emulsion" usually resulted in a temporary but marked decrease in the glycemia and glycosuria. The first few experiments were briefly reported in 1915.[6] The present paper includes these experiments as well as a large number of experiments performed since the first communication.

Method.

Operation.—Total depancreatization was performed in the majority of cases. In three animals, used in the six most recent experiments, the "Allen" procedure, *i.e* leaving a small remnant around the large pancreatic duct, was adopted The operations were performed under ether anesthesia.

Preparation of "Emulsion".—Fresh dog's pancreas was hashed, mixed with three or four times its weight of sterile distilled water, and placed in the refrigerator. After a period of from 1 to 20 hours it was strained and squeezed through muslin. The fluid thus obtained was diluted with 5 volumes of sterile 0.9 per cent NaCl solution before injection. This dilute solution was faintly acid or neutral to litmus, light pink in color, and almost clear. At no stage was it filtered, nor was sodium carbonate or any other substance added. In one experiment (LP77a), Ringer's solution was used in the extraction and also in the dilution; this did not lead to a better result. The emulsions of other tissues, used in the control experiments, were prepared in a similar manner.

Injection and Blood Sampling.—Some time before the infusion was to be given, the animals (in most of the experiments) received a small dose of morphine sulfate subcutaneously, usually about 1.5 mg. per kilo Cannulas were then introduced under local anesthesia into a convenient vein and artery, for injection and blood sampling, respectively. In the six recent experiments no morphine was given and only one cannula (the venous) was introduced under local anesthesia (ethyl chloride). In this series

the blood was drawn directly from an external jugular vein into a syringe. The urine was obtained by catheter in all cases.

Analytical Methods —The blood sugar determinations were made by the Myers-Bailey[10] modification of the Lewis-Benedict method. Glucose in the urine was estimated by a modification of the Pavy[11] method or by Benedict's[12] method. Hemoglobin was determined in most of the experiments by discharging 2 cc. of blood into dilute HCl (about 0.3 per cent) in a 250 cc volumetric flask, diluting to this volume, and comparing the density of the color in a Duboscq colorimeter The first blood sample of each experiment was used as the standard and was taken as 100 per cent for that experiment Later the Sahli hemoglobinometer was used and, to avoid confusion, the results are similarly expressed.

Four illustrative protocols, including three injections of pancreas emulsion and one control, follow.

Experiment LP72a —From Table I it will be observed that the high blood sugar value of 0.31 per cent fell to 0 14 per cent in the course of the 78 minute injection period and after another period of 90 minutes it was still low (0 13 per cent). Both of these figures, 0 14 and 0.13 per cent, are practically normal values, but emphasis is to be placed on the extent of the drop, from 0.31 to 0.13 per cent, a fall of 0.18 per cent, rather than upon the absolute value finally obtained At the same time this decrease in the concentration of the blood sugar is not accompanied by any change in the hemoglobin value, or by an increased glycosuria. In other words it is not due to a dilution of the blood sugar by the injection fluid, or to a washing out of the sugar through the kidneys. In fact the kidney excretion of sugar is greatly diminished.

This was one of the few experiments in which the injection produced any noticeable general symptoms; there was first a fall and then a rise in temperature. There was no marked harmful effect, of the infusion, however, as the animal was given a second infusion 2 days after the first and lived 2 days after that The second infusion caused only a slight fall in the glycemia (see LP72b in Table V)

[10] Myers, V. C , and Bailey, C. V , *J Biol Chem* , 1916, xxiv, 147
[11] To be published shortly
[12] Benedict, S R , *J Biol Chem* , 1911, ix, 57

TABLE I

Experiment LP72a

Male, 11 25 Kilos, Completely Depancreatized April 19, 1915.

Date	Time		Blood sugar	Hemoglobin per cent of first sample	Urine			Temperature
					Volume	Glucose		
			per cent		cc	per cent	gm per hr	°C
	a m							
Apr. 20	10 00	Injected 1.5 mg of morphine sulfate per kilo subcutaneously						
	10 23							39 1
	10 36	Cannulas have been introduced into left external jugular vein and right carotid artery under ethyl chloride and cocaine hydrochloride						
	11 08				20	10 08		
	11 11		0 31	100				
	11 15– 12 33	Injected intravenously 112 cc of dilute pancreas emulsion prepared from the pancreas of this dog						
	11 23	Cheyne-Stokes respiration during most of injection						
	p m.							
	12 34		0 14	101				37 9
	12 44				31 5	8 79	1 85	
	12 48							38 8
	1 00	Slight tremors of muscles of head and hind legs						
	1 18							40 3
	1 35	Tremors have stopped						
	2 04		0 13	100				
	2 15				42 5	1 62	0 46	
	2 18							40 8
	a m.							
Apr. 21	9 45	Not catheterized			193	1 49		39 3

Experiment LP76 —In Experiment LP76 (Table II), in spite of an intense hyperglycemia and glycosuria the administration of

TABLE II

Experiment LP76

Female, 9 Kilos, Completely Depancreatized May 11, 1915

Date	Time		Blood sugar	Hemoglobin in per cent of first sample	Urine			Temperature
					Volume	Glucose		
	a m		*per cent*		*cc*	*per cent*	*gm per hr*	*°C*
May 12	10 15							38 5
	10 40	Injected 1 4 mg of mor-phine sulfate per kilo subcutaneously						
	11 06	Cannulas have been in-troduced into left ex-ternal jugular vein and left carotid artery under ethyl chloride and cocaine hydro-chloride						
	11 07	Catheterized.			111	+++		
	11 20							38.0
	11 43				20 5	11 16	3 82	
	11 45		0 33	100				
	11 49–	Injected intravenously						
	12 52	112 cc dilute pancreas emulsion prepared from the pancreas of this dog						
	p m							
	12 53				30	12 38	3 18	
	12 54		0 25	101				
	1 13							39 0
	2 27				14	3 64	0 33	
	2 29		0 23	103				
	2 43							39.5

pancreatic emulsion reduced the blood sugar and urine sugar to a marked degree, (0.10 per cent), without producing any unfavor-able symptoms or affecting the temperature very much.   2 days

later a different type of experiment was performed on this animal, after which it was killed with chloroform.

Experiment DP1a.—Experiment DP1a (Table III) was one of the more recent ones. No morphine was used and only an in-

TABLE III

Experiment DP1a.

Female, 8 4 Kilos, 92 Per Cent of the Pancreas Removed on April 7, 1919, the Remnant Left Being in Communication with the Large Duct

Date	Time		Blood sugar	Hemoglobin in per cent of first sample	Urine			Temperature
					Volume	Glucose		
	a m		*per cent*		*cc*	*per cent*	*gm per hr.*	*°C*
Apr. 8	10 20		0 20	100				
	10 36	Catheterized				1 25		
	11 19							38 8
	11 30	Under ethyl chloride a cannula has been inserted in the right external jugular vein.						
	11 33		0 21	102				
	11 43				16	8 0	1 15	
	11 49–	Injected intravenously						
	12 31	60 cc. of dilute pancreas emulsion prepared from the pancreas of this dog						
	p m							
	12 36		0 12	102				
	12 44				10	5 40	0 53	
	2 15		0 08	96				
	2 23				14	0 5	0 04	
	2 26							40 4
	a m							
Apr 9	10 08		0 27	99	800	0 33 ⎫	0 20	
	10 20	Catheterized			20	3 62 ⎭		
	10 26							38 5

jection cannula was introduced into a vein   The animal was kept on the board only during the necessary experimental procedures   The infusion of pancreas emulsion in this case caused no unfavorable symptoms and had a very striking effect on the glycemia

Before injection there was a constant and moderate hyperglycemia (0.20 and 0.21 per cent). At the end of the injection, the blood sugar had fallen to 0 12 per cent, practically a normal figure, and 1 hour and 39 minutes later the distinctly normal value, 0.08 per cent, was found On the next morning hyperglycemia had again been established. The urinary sugar record is also of interest.

TABLE IV.

Experiment LP81a.

Male, 14 25 Kilos, Depancreatized June 7, 1915, at least 94 Per Cent of the Pancreas Removed

Date	Time		Blood sugar	Hemoglobin in per cent of first sample	Urine			Temperature
					Volume	Glucose		
	a m		*per cent*		*cc*	*per cent*	*gm per hr*	°C
June 8	10 35	Injected 1 5 mg of morphine sulfate per kilo subcutaneously						
	11 19	Cannulas have been introduced into the left carotid artery and right jugular vein under ethyl chloride						
	11 24							37 8
	11 43	Catheterized			116	5.96		
	11 52		0 34	100				
	11 56–	Injected intravenously						
	12 39	114 cc of dilute emulsion of normal dog's						
	p m	submaxillary gland						
	12 41		0 32	95				
	1 08							38 3
	2 14		0 33	94				
	2 38				100	3 32	1 14	

The excretion of 1.15 gm. of glucose per hour before the injection was followed by a diminution to half that figure during the injection and thereafter only traces were excreted for at least an hour and a half (0.04 gm. per hour). The over night urine also contained very little sugar (0.2 gm. per hour) but it is evident that the kidney was not rendered permanently impermeable to

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sugar because the next urine obtained by catheter contained
3 62 per cent dextrose.

On the following day (see DP1b in Table V) an injection of
another pancreas preparation lowered the blood sugar from 0.27
to 0 20 per cent.   The urinary sugar excretion was diminished
very markedly; in one period no qualitative reaction whatever
could be obtained.

Experiment LP81a.—In Table IV is given an outline of Experi-
ment LP81a, one of the control experiments.   The animal received
an intravenous injection of submaxillary gland emulsion.   The
slight decrease in the blood sugar percentage is in marked contrast
with the results obtained when pancreas emulsion was used, and
runs parallel with the hemoglobin, *i e* , here what little effect there
is may be referred to dilution by the intravenous injection of 114
cc. of fluid.   The urinary data are insufficient in this experiment
but indicate a possibly diminished output of sugar

There were no noticeable general effects caused by the infusion
and *on the next day the administration of pancreatic emulsion
lowered the blood sugar from 0 38 to 0 24 per cent* (see LP81b, Table
V).

In Table V all the experiments with pancreas emulsions are
collected   It will be seen that in most of them there occurred
a substantial reduction in the blood sugar.   As the samples were
not taken at short intervals it is quite probable that the lowest
values were not discovered.   However, normal or nearly normal
figures were found in several cases   Study of the table indicates
that, when effective, the emulsion produces an effect during the
slow injection, because the blood sugar is usually lower at the
end of the injection than at the beginning.   The glycemia remains
low or continues to fall for about 1½ to 1¾ hours, and then, if we
may trust the few figures at hand, it gradually rises   It is certain
that the effect does not last many hours, for in a number of the
experiments a sample of blood was taken on the following morning
and invariably showed a hyperglycemic value.

In all except one experiment (LP75), there occurred a dimi-
nution in the excretion of sugar.   In some cases this was quite
marked, resulting in a urine reacting negatively to Benedict's
qualitative test.   Such urines, however, had normal color and
specific gravity.   In about half the experiments there was a

TABLE V.

Injection of Pancreatic Emulsions into Depancreatized Dogs

Experiment No	Days after operation	Blood sugar				Sugar output in urine per hour					Emulsion prepared from pancreas of same or different animal	Remarks
		Before injection	End of injection	After injection		Before injection	During injection	After injection				
		per cent	per cent	mm	per cent	gm	gm	hrs	gm	cc.		
LP70	2	0 28	0 09	92	0 08		0 92	1 5	Tr	1 4	Different	This dog used for other experiments on preceding day and again 5 days later
LP72a	1	0 31	0 14	91	0 13		1 85	1 50	46	42 5	Same	Slight symptoms produced by injection (see protocol, Table I) Used 2 days later for Experiment LP72b
LP72b	3	0 28	0 22	93	0 22		0 88	1 40	61	15	"	No symptoms
LP73	2	0 33	0 25	71	0 16		↑	0 8	+	5*	"	Dog died 50 min after end of injection, heart blood obtained 21 min later Peritonitis, perhaps 25 per cent of pancreas not removed
LP74a	1	0 27	0 21	93	0 18		5 07	1 60	66	9	"	Used 4 days later for Experiment LP74b
LP74b	5	0 23	0 18	92	0 13		1 54	1 50	07	5 5	Different	Dog used on the next day for another experiment
LP75	2	0 27	0 22	101	0 23		1 14	1 81	55	34	"	Killed 3 days later; about 8 per cent of pancreas not removed
LP76	1	0 33	0 25	97	0 23	3 82	3 20	1 60	32	14	Same	Used this dog 2 days later for another experiment
LP77a	1	0 21	0 17	95	0 16	1 26	0 66	1 60	05	23	"	Ringer's solution used in preparing emulsion, pregnant animal, used 6 days later for LP77c
LP77c	7	0 24	0 25	90	0 22		↑	1 50	2*	23 5*	Different	Dog used 6 days before for LP77a, and 4 days before for LP77b (Table VI)

												Remarks
LP81b	2	0 26	0 38 •	90	0 24		1 79	1 7	0 09	22 5	Different	Received injection of submaxillary emulsion on preceding day (Experiment LP81a, Table VI), killed 2 days later, perhaps 6 per cent of pancreas not removed
DP1 a†	1	0 20 / 0 21	0 12	104	0 08	1 16	0 54	1 7 / 20	0 04 / 0 17	14 / 820	Same	"Allen" procedure, 8 per cent of pancreas left, used 2 days later for Experiment DP1b
DP1 b†	3	0 25 / 0 27	0 21	94 / 240	0 20 / 0 21	>1 15	1 25	2 2 / 1 9 / 18	0 00 / 0 08 / 0 44	60 / 65 / 260	Different	Killed on following day.
DP2 a†	2	0 31 / 0 30	0 24	107	0 19	1 00	0 88	1 7 / 18	0 04 / 0 00	38 / 425	Same	"Allen" procedure, 4 per cent of pancreas left, used 3 days later for Experiment DP2b
DP2 b†	5	0 30 / 0 33‡	0 26	81 / 164	0 17 / 0 24	1 20	1 34	1 1 / 1 4 / 18 5	0 00 / 0 42 / 0 55	26 / 83 / 583	Different	Had been used 3 days before for Experiment DP2a, used 3 days later for Experiment DP2c
DP2 c†	8	0 33	0 29	140 / 246	0 26 / 0 30	1 85 (24 hrs)	0 26	2 0 / 1 9	0 00 / 0 25	39 / 66	Same	Had been used for Experiments DP2 a and b

* During and after injection.
† No morphine given
‡ After feeding

TABLE VI

Injections of Emulsions of Tissues, Other than Pancreatic, into Depancreatized Dogs

Experiment No.	Days after operation	Blood sugar			Sugar output in urine per hour					Tissue used for emulsion	Remarks.
		Before injection per cent	End of injection per cent	After injection min / per cent	Before injection gm	During injection gm	After injection hrs	gm	cc		
LP77 b	3	0 23	0 26	92 / 0 28	1 07	↑	1 8	0 15*	23 5*	Submaxillary gland	Had been used 2 days before for Experiment LP77a, used 4 days later for Experiment LP77c (see Table V), pregnant
LP81 a	1	0 34	0 32	95 / 0 33		↑	2 0	1 14*	100*	"	Used on the following day for Experiment LP81b (Table V), perhaps 6 per cent of pancreas not removed
LP84	1	0 31	0 31	92 / 0 36		↑	1 8	0 08*	2 2*	"	Required 4 2 mg of morphine per kilo; considerable depression, died during night, cause of death could not be found at autopsy, 4 per cent of pancreas not removed
LP86	1	0 39	0 37	92 / 0 42	1 9	→	1 8	0 27*	11*	"	Killed on following day
LP83	1	0 41	0 36	101 / 0 35		4 75	1 7	2 12	28	Spleen	Died 3 days later of pulmonary edema
DP3†	5	0 31	0 32	114 / 0 32		0 28	3 0	0 20	26	Voluntary muscle.	"Allen" type, at time of this experiment glycosuria was not marked, had been used 4 days before for another experiment, chloroformed 6 weeks later, no morphine used
				193 / 0 32			21 5	>1 9	830		

*During and after injection
†No morphine given.

decided reduction in the volume of urine secreted in the 1½ hour period following the injection, indicating perhaps some temporary functional damage to the kidney; it is possible that this may be caused by the injection of any tissue extract. In the rest of the experiments there was no diminution in urinary flow even when the sugar output had practically ceased (see Experiments LP77a, DP1 a and b, DP2 a, b, and c, Table V).

The controls in which emulsions of several other tissues were injected are tabulated in Table VI. There was no marked effect

TABLE VII.

Summary

Maximum Reduction of Blood Sugar Produced by Injection of Tissue Emulsion.

Marked reduction			Moderate or no reduction		
Experiment No	Maximum reduction of blood sugar	Kind of emulsion	Experiment No	Maximum reduction of blood sugar	Kind of emulsion
LP70	0 20	Pancreas.	LP72b	0 06	Pancreas
LP72a	0.18	"	LP75	0 05	"
LP73	0 17	"	LP77a	0 05	"
LP74a	0 09	"	LP77c	0 02	"
LP74b	0 10	"	DP1b	0 07	"
LP76	0 10	"	DP2c	0 07	"
LP81b	0 14	"	LP77b	Increase	Submaxillary gland.
DP1a	0 13	"	LP81a	0 02	" "
DP2a	0.11	"	LP84	Increase	" "
DP2b	0 16	"	LP86	0 02	" "
			LP83	0 06	Spleen.
			DP3	Increase	Muscle.

on the blood sugar in any of these tests. Injection of emulsion of spleen (Experiment LP83) was followed by a slight fall of blood sugar, from 0.41 to 0.35 per cent; this was the maximum effect observed in any of the controls. Indeed a slight increase in the blood sugar was observed in three experiments. The amount of sugar eliminated by the kidney was usually decreased to some extent, but not so much as in the pancreas injection experiments. In none of these controls, for instance, was a negative or weak Benedict's test obtained and it is probable that in some of these, as in many of the pancreas emulsion experi-

ments, we have to do with an impairment of the renal epithelium. The striking point in these six experiments is the regular failure to observe a marked lowering of the blood sugar as contrasted with the success when pancreas emulsion was used

This difference is brought out in the summary (Table VII). Here it will be observed that ten out of sixteen pancreas emulsion injections resulted in a marked reduction of the blood sugar, the greatest reduction in each case falling in the range 0.09 to 0.20 per cent. The other six pancreas experiments, as well as all the controls, showed a reduction of only 0 07 per cent or less. Only one of all the sixteen pancreas experiments had practically a negative result (LP77c), while only one of the controls showed anything resembling a positive effect (LP83).

DISCUSSION.

Many investigators[13] have recognized that the best evidence for the internal secretion theory of the origin of diabetes would be an antidiabetic effect of a pancreatic preparation, administered parenterally. The experiments just described show that such a result has been obtained, since the reduction of hyperglycemia is surely an antidiabetic effect, and the reduction of glycosuria is possibly partly of the same nature The demonstration of the actual combustion of sugar has not been attempted although the additional proof, while not absolutely essential, would be welcome. A favorable effect upon the glycemia and glycosuria has, however, been established and there remain only a few points to be considered.

The reduction in glycemia is real, that is, it is not due to dilution. The hemoglobin values, given in the protocols, indicate that this is the case; the same holds true for all the pancreas injection experiments, although this has not been recorded in Table V. Furthermore, that it is not an accident, a mere coincidence, is proven by the fact that it occurred to a marked degree in ten out of sixteen experiments, and to some extent in fifteen out of sixteen. The controls include only one with any effect— and that an insignificant one—on blood sugar. In some cases

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[13] See, for example, Hédon, E , Travaux de Physiologie, Paris, 1898, 133 Pfluger, E , *Arch ges Physiol* , 1907, cxviii, 271

the pancreas emulsion injection resulted in changing a pronounced hyperglycemia to a normal blood sugar. It is quite likely that more of these cases occurred than were discovered, because the blood samples were not taken at short intervals.

No increased glycosuria occurred which could be said to account for the decrease in blood sugar. On the contrary, the elimination of sugar was decreased in every experiment except one, this being one in which the effect on the blood sugar was slight (Experiment LP75, Table V). The effect upon the urinary sugar generally lasted longer than that upon the blood sugar, but specimens of urine obtained by catheterization on the day following the injection usually contained high percentages of sugar. This would indicate that if the injection of an organ extract decreases the permeability of the kidney to sugar by a toxic action, the effect is only temporary. Since in the controls a similar, but less marked, transitory reduction of the urinary sugar output was observed, it is evident that *the mere reduction of glycosuria is no proof of a beneficial effect of any agent*. Observations of other investigators may be cited in this connection. Murlin and Kramer[5] in one experiment found that an intravenous injection of a mixed boiled extract of pancreas and duodenal mucosa produced a marked fall in the sugar output of a diabetic dog, but that there was a *rise* in the blood sugar. Hédon[14] has shown that transfusion of normal blood into diabetic animals may result in a diminished output of sugar with little if any reduction of the blood sugar level. He assumes that the diabetic complex consists of two phases, of which the renal is the first to be affected by the internal secretion of the pancreas. Is it not possible, however, that the introduction of a foreign blood had a toxic effect on the kidney? In fact Hédon himself admits that cross circulation between two diabetic dogs also diminishes glycosuria. The same criticism applies to all blood transfusion and related investigations[15, 16] in

[14] Hédon, E, *Compt rend Soc biol*, 1911, lxxi, 124

[15] Forschbach, J, *Arch exp Path u Pharmacol*, 1908–09, lx, 131   This author united animals of the same litter (parabiosis) and later removed the pancreas of one, without causing more than a mild glycosuria   In one experiment (No 5, p 145) he reports a blood sugar of 0 32 per cent while the urine was sugar-free

[16] Drennan, F M, *Am J Physiol*, 1911, xxviii, 396

which only the urine was examined. The same question might be asked regarding Scott's work,[4] for he, too, used only the urinary output as an index of the action of his pancreatic extract.

It must be emphasized that the beneficial effects of the pancreas emulsion are not to be referred to sodium carbonate or any other alkali. *No alkali was used in preparing the pancreas emulsions.* The extractions were made with water and the dilutions of the extract, or emulsion, with 0.9 per cent NaCl. The resulting injection fluid was faintly acid to litmus. Our experiments, therefore, cannot be compared with Murlin and Kramer's [5],[7] because they always used an alkaline medium. The alkalinity itself is, according to these authors, largely responsible for the beneficial action of the various tissue extracts which they administered In our experiments, on the other hand, the favorable effect of the pancreatic emulsion must be due to some ingredient peculiar to it, because no alkali was used in its preparation, and because the other tissue emulsions, prepared in a similar manner, had no such favorable effects

The mechanism of the effect on the blood sugar may be considered briefly. There are several possibilities, corresponding to the several hypotheses for the mechanism of diabetes. The author does not wish to enter here into an extended discussion of these hypotheses, but wishes to point out how the results of this investigation apply in each case. For the "overproduction" theory the injection fluid may be held to contain a substance which decreases the output of sugar by the liver. If one believes that, in diabetes, the cells themselves lack the power to burn sugar—as many investigators do—it will be said that the pancreas emulsion contains the substance which stimulates the cells to this activity, or the "amboceptor" (Allen) which enables the cells to anchor sugar to their protoplasm.

Kleiner and Meltzer[17] have indicated another explanation for some of the phenomena of diabetes; *i.e.,* a decreased permeability to sugar of the capillary endothelia and perhaps of other cells as well. Palmer[18] has corroborated this by showing that the striated muscle of diabetic animals has a lower concentration of glucose

[17] Kleiner, I S , and Meltzer, S J., *Proc Soc Exp Biol. and Med.,* 1914-15, xii, 58

[18] Palmer, W W , *J Biol Chem ,* 1917, xxx, 79.

than that of normal animals, if the respective levels of blood
sugar are·taken into account. We have also reported that pancreas emulsions enable diabetic animals to handle, in a nearly
normal manner, large amounts of intravenously administered
dextrose. This was taken to mean that the pancreas emulsion
had restored the permeability, thus permitting the sugar to be
absorbed and taken care of in the normal manner. The same
explanation may be applied to the present experiments. In other
words the pancreas emulsion perhaps contains some substance
which alters the permeability of the capillary walls, allowing the
diabetic sugar to go through them into the tissues. Another
factor which may come into consideration is the state of the
blood sugar. The author has presented evidence[19] that the
diabetic blood sugar is in a combined or poorly diffusible state.
This indicates a further difficulty presented to the diabetic organism in its struggle to obtain sugar for its starving tissues. The
enzymes present in the pancreas emulsion may be able to break
up this combination setting free crystalloid glucose. This can
diffuse readily through the tissues which at the same time have
been rendered more permeable to it. This last possibility may
be tested directly by the method already described.[19]

The question of the general toxicity of the extracts used requires
a few words. It might be expected that an unfiltered tissue
extract of this sort would produce grave symptoms when injected
intravenously. This was not the case, however. The explanation seems to be that the extract was highly diluted and very
slowly injected. In but few instances among the entire list of
experiments was any depression or other unfavorable symptom
observed. Furthermore, most of the animals received several
infusions on different days and survived them One dog died
shortly after a pancreas emulsion injection, but death was apparently due to peritonitis. One control died a few hours after the
injection of submaxillary emulsion, and another died of pulmonary edema 3 days after spleen emulsion had been administered.

The fact that these pancreas emulsions lower blood sugar in
experimental diabetes without causing marked toxic effects indicates a possible therapeutic application to human beings. It is

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[19] Kleiner, I S , *J Biol. Chem*, 1918, xxxiv, 471

true that the numerous attempts at pancreas therapy have been almost uniformly unsuccessful. However, the methods here-tofore used have not resembled the one used in our experiments. This method is based on certain principles, discussed early in this paper, and is extremely simple. It is possible that the tem-porary effect which it produced in dogs might be duplicated in man and might be useful in certain emergencies. However, it is doubtful whether attempts along this line should be made until further knowledge has been obtained. It is important to know whether a filtered extract would be effective, and particularly whether an emulsion of the pancreas of another species would have its effect when injected into a diabetic dog. Other ques-tions also arise. Do such injections raise the respiratory quo-tient? Could other paths of injection and other animals be used equally well? Finally the search for the effective agent or agents, their purification, concentration, and identification are sug-gested as promising fields for further work.

SUMMARY.

Diabetic dogs were given intravenous injections of unfiltered water extracts of fresh pancreas, diluted with 0.9 per cent NaCl solution. The preparation was administered very slowly and usually resulted in a marked decrease in the blood sugar. There was no compensating increase in urinary sugar, but rather a decrease, which may be partly due to a temporary toxic renal effect.

The result is regarded as further evidence for the internal secretion theory of experimental diabetes.

CRYSTALLINE GUANYLIC ACID.

By P. A LEVENE

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The theory of the polynucleotide structure of the yeast nucleic acid has been receiving confirmation in recent years through the investigations of several workers. Levene,[1] Jones and Kennedy,[2] and Thannhauser and Dorfmuller[3] have described crystalline mononucleotides obtained from yeast nucleic acid   The present communication contains a report on guanylic acid obtained in crystalline form.   It crystallized in the form of long prismatic needles having the same appearance as guanosine   The substance had all the properties of guanylic acid.   It gelatinized in the presence of mineral impurities.   A test for the presence of free phosphoric acid was negative.   It had no melting point but turned brown at 208°C.   The optical rotation in water solution was $[\alpha]_D^{20} = -7.5$, and in a 5 per cent ammoniacal solution $[\alpha]_D^{20} = -43\ 5$. On hydrolysis it gave guanosine or guanine sulfate depending on the conditions of the experiment   The free acid, when air-dry, crystallized with 2 mols of crystal water, and, when dried under diminished pressure at the temperature of a toluene vapor bath to constant weight, still retained $\frac{1}{2}$ mol of crystal water   The pure guanylic acid was converted into a crystalline brucine salt.

EXPERIMENTAL

Crude brucine salt of guanylic acid was converted into the ammonium salt in the manner described in a previous publication.[4] The ammonium salt was dissolved in hot water.   The resulting

[1] Levene, P. A , *Proc Soc Exp Biol and Med* , 1917–18, xv, 21

[2] Jones W , and Kennedy, R P , *J Pharmacol and Exp Therap* , 1919, xii, 253

[3] Thannhauser, S J , and Dorfmuller, G , *Z physiol Chem* , 1919, civ, 65.

[4] Levene, P. A , *J Biol Chem* , 1919, xxxix, 77

solution was acidulated, and the substance precipitated by means of neutral lead acetate. The precipitate was repeatedly washed with water, then suspended in water, and freed from lead. The resulting solution was again precipitated with neutral lead, and the process repeated once more. Finally the solution obtained on decomposition of the lead salt gave on concentration under diminished pressure a crystalline deposit.

On drying under diminished pressure at the temperature of xylene vapor, the substance still retained $\frac{1}{2}$ mol of crystal water.

0 1084 gm. of the vacuum-dry substance gave 0 1274 gm of CO_2 and 0 0424 gm of H_2O

0.1834 gm. of the substance used for Kjeldahl nitrogen estimation required 24 45 cc of 0.1N acid for neutralization.

0 2757 gm of the substance gave 0 0844 gm of $Mg_2P_2O_7$.

							Calculated for $C_{10}H_{14}N_5O_7+\frac{1}{2}H_2O$	Found
							per cent	per cent
C	.	.	. .	.	...	.	32 24	32 05
H			.	. . .	.		4 03	4 38
N .	.		..		.	.	18 80	18 67
P	..		.	..		.	8 33	8 55

In 10 per cent hydrochloric acid the substance was optically inactive. In aqueous solution the rotation was

$$[\alpha]_D^{20} = \frac{-0\ 15 \times 100}{1 \times 2} = -7\ 5$$

In 5 per cent aqueous ammonia solution the rotation was

$$[\alpha]_D^{20} = \frac{-0\ 87 \times 100}{1 \times 2} = -43.5$$

· *Acid Hydrolysis of the Substance.*

2.0 gm. of the substance were dissolved in 20 0 cc of 2 per cent sulfuric acid and boiled over a flame with a return condenser for 1 hour. The substance was filtered and allowed to cool. Soon a crystalline deposit formed. This was filtered and recrystallized out of 5 per cent sulfuric acid. The substance was dried over night over sulfuric acid in a vacuum desiccator.

0 0905 gm of dry substance gave 27 6 cc of nitrogen gas at $T° = 25°$ C and $P_o = 763$.

	Calculated for $(C_5H_5N_5O)_2H_2SO_4$ per cent	Found per cent
N	35 00	35 08

Ammonia Hydrolysis.

2.5 gm. of the substance were dissolved in 50 cc. of water containing 5 cc of ammonia water, and the solution was made up to 75 cc. It was then heated in a sealed tube for 4 hours at 135°C. On cooling, the contents of the tube practically solidified. The solid material was filtered on suction and the residue recrystallized twice out of water. The crystals had the characteristic appearance of guanosine. The yield of the recrystallized substance was 0.6 gm.

0 100 gm of the substance employed for Kjeldahl nitrogen estimation required for neutralization 8 8 cc. of 0 1N acid

	Calculated for $C_{10}H_{13}N_5O_5 \cdot 2H_2O$ per cent	Found per cent
N	21 94	21 98

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{-0 66 \times 100}{1 \times 1} = -66.0$$

Since this rotation was slightly higher than the one previously recorded (-60.5), and since in the earlier work a less sensitive instrument was employed for the measurement, the rotation of a pure sample of guanosine was measured under exactly the same conditions as the sample obtained in the present work. The value obtained was

$$[\alpha]_D^{20} = \frac{-0 66 \times 100}{1 \times 1} = -66 0$$

Brucine Salt.

2 gm. of the crystalline guanylic acid were dissolved in water and neutralized by means of a solution of brucine in methyl alcohol. A crystalline deposit formed immediately. This was

filtered off and recrystallized out of a 35 per cent solution of alcohol in water. The air-dry substance contracted at 217° (Anschutz thermometer), melted into a brown liquid at 233°, and effervesced at 240°C.

0 1000 gm. of the substance gave on combustion 0 1938 gm of CO_2 and 0 0558 gm of H_2O

0 2000 gm of the substance gave 18 2 cc of nitrogen gas at $T° = 25°$ and $P = 759$

0 300 gm of the substance gave 0 262 gm of $Mg_2P_2O_7$

				Calculated for $C_{56}H_{84}N_9O_{16} \ 7H_2O$	Found
				per cent	per cent
C	.	. .		52 61	52 85
H		. .		6 78	6 24
N	.	.		9 88	10.41
P	.	. .	.	2 43	2 43

The rotation of the substance in 35 per cent alcohol solution was

$$[\alpha]_D^{20} = \frac{-0\ 52 \times 100}{1 \times 2} = -26\ 0$$

A NEW STEROL.

By TAKEO IKEGUCHI

(From the Medical-Chemical Institute of the Medical College of Osaka, Japan)

The sterols are probably contained in all cells, and there seems to be no doubt of their importance physiologically. Until now, however, the only property which has been conclusively proved is their antidotal effect toward the hemolytic action of the saponins.

If we take into account the poisonous action of some of the sterol derivatives upon the tissues of the heart, the biological significance of sterol affords an interesting field for research.

Whereas cholesterol, which is commonly found in animals, has received much attention, and its structure has been practically confirmed by Mauthner, Abderhalden, and especially by Windaus,[1]
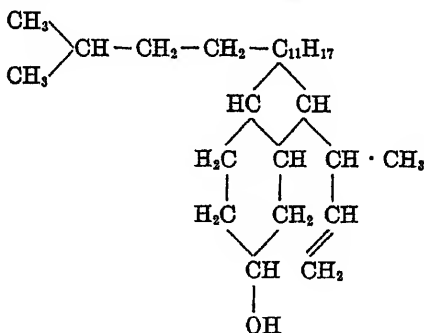
the other members of the sterol group, which occur in plants and fungi, have received but slight consideration.

Several varieties of sterol have been isolated from fungi. Tanret[2] obtained ergosterin and fungisterin from ergot, and Zellner[3] separated a sterol, with the formula $C_{26}H_{40}O$, from the

[1] Windaus, A , *Ber chem Ges* , 1912, xlv, 2421

[2] Tanret. quoted by Windaus, A , in Abderhalden, E , Biochemisches Handlexikon, Berlin, 1911, iii, 309

[3] Zellner, J , quoted by Fodor-Halle, A , in Abderhalden, E , Biochemisches Handlexikon, Berlin, 1914, viii, 493

toadstool. Up to the present, however, no one appears to have separated one identical sterol from different species of fungi, comparing it with other sterols and determining its structure. This is the reason for attempting investigation along this line.

In an earlier treatise,[4] the writer described a sterol-like subtance which he obtained from the *Lycoperdon*. During the course of the present research, he examined *Collybia shiitake*, from which he isolated another sterol in pure form. This he traced through *Armillaria edodes, Hydnum asparatum*, and *Lycoperdon gemmatum*.

Method of Preparation and General Tests.

100 gm. portions of each of the above mentioned fungi, dried as well as possible at room temperature, and then thoroughly in an oven at 70°C., were extracted with ether   The ether was then distilled, leaving in each case an oily residue, which soon coagulated into a cheesy mass containing the sought for crystals of sterol. The crystals were washed with petroleum ether and alcohol, and then recrystallized from a hot solution of the latter solvent. Each batch of fungi, treated in this manner, yielded about 0.1 gm. of long, colorless, hexagonal crystals.

All the crystals obtained gave the same color reactions. (1) The crystals were dissolved in chloroform; an equal volume of concentrated sulfuric acid was then carefully added so as to form a separate layer. From the contact surface between the two layers the lower sulfuric acid layer gradually became blood-red, while the chloroform layer turned brownish blue, there being a distinct green fluorescence in both layers. (2) To a chloroform solution of the crystals a little acetic anhydride and two drops of concentrated sulfuric acid were added   The mixture became at first rose-red, then successively blue, and dark green. (3) The crystals were dissolved in acetic anhydride. On adding a few drops of concentrated sulfuric acid the mixture turned first rose-red, then violet, then blue, and finally green. (4) The crystals do not give the oxycholesterol reaction.

Each of the above reactions is similar to the corresponding one for cholesterol. In the first test, however, where cholesterol is

[4] Ikeguchi, T , Z *physiol. Chem.*, 1914, xcii, 257.

concerned, the coloration shown by the two layers is reversed. It resembles the cholestanon[5] reaction, but can be differentiated from the latter by the difference of color of the chloroform layer.

The crystals obtained, as indicated above, by the extraction of the various fungi are colorless and odorless and melt at 159–160°C. When exposed to the air for a long time or heated above 80°C., they become yellow in color. On ignition they emit an odor resembling that of isovaleric acid. They are readily soluble in ether, chloroform, and warm alcohol, with difficulty in cold alcohol and petroleum ether, and are insoluble in water.

In solution the substance is levorotatory The sample to be determined was dissolved in chloroform and the measurements were made with a Landolt polariscope, using a 2 dm. tube. The specific rotation of the substances obtained from each fungus was nearly the same

Name of fungus	Specific rotation $([\alpha]_D^{20})$.
Collybia shiitake	−129 4
Armillaria edodes	−129 23
Hydnum asparatum	−129 55

and the results obtained by analysis were also nearly equal

Name of fungus	Weight of sample gm	CO_2 gm	H_2O gm
Collybia shiitake	0.1464	0 4402	0 1464
Armillaria edodes	0 1086	0 3260	0 1110
Hydnum asparatum	0 1568	0 4746	0 1556

Found		Calculated $(C_9H_1O_2)$	
C per cent	H per cent	C per cent	H per cent
82 004	10 81	81 81	10 9
81 87	11 35		
82 02	10 95		

The yield of the substance obtained from the *Lycoperdon* was so small, owing to the difficulty of isolating it from other materials present, it could not be analyzed, however, there is no doubt that it is identical with others, agreeing with them in its crystal form, color reactions, and melting point.

[5] Diels, O , and Abderhalden, E , *Ber chem Ges* , 1904, xxxvii, 3099

The sterol obtained probably occurs not only in the fungi above mentioned but throughout all classes of fungi, wherefore I shall name the compound mycosterol.

According to the analytical results, mycosterol may be regarded as an oxidation product of stigmasterol,[6] and this, referring to the fact ascertained by Lifschutz that cholesterol is converted into oxycholesterol in the animal body, gives mycosterol an added interest.

In order to ascertain whether mycosterol is capable of preventing the hemolytic action of saponin, an experiment was carried out following Hausmann's method.[7] 5 cc of an ether solution of mycosterol were gradually added with stirring to 5 cc. of saponin solution (0 1 gm of saponin in 100 cc. of isotonic sodium chloride solution).   The mixture was placed in an oven at 40°C. for 7 to 8 hours, then at 30°C. for 1 to 2 hours, after which the ether evaporated *in vacuo.* 1 and 2 cc. of the mixture thus prepared were added respectively to two 5 cc. portions of a floating solution of red corpuscles taken from a rabbit   The phenomenon of hemolysis occurred to a small extent in 24 hours, while a similar blood solution to which the saponin solution only had been added showed complete hemolysis instantaneously.   Another similar solution with cholesterol in place of the mycosterol showed no hemolysis at all even after 24 hours

This experiment shows that mycosterol, as well as cholesterol, is an antidote against saponin though its power is not so great.

Digitonin Compound [8]—Mycosterol was dissolved in boiling alcohol, and a 1 per cent alcohol solution of digitonin was added in excess until the mixture presented a slight turbidity.   After standing for about 1 hour, crystals separated out which were filtered off, washed with alcohol and ether, and then recrystallized from methyl alcohol after adding a little water.   The crystals melted at 242°C. with the evolution of gas.

The crystals are soluble in pyridine, and with difficulty in acetone and benzene.   They give the typical Liebermann-Burhard reaction which serves to show that the crystals are a compound of mycosterol and digitonin.

[6] Windaus, A , and Hauth, A , *Ber chem Ges* , 1906, xxxix, 4378.

[7] Hausmann, W , *Beitr. chem Physiol u Path* , 1905, vi, 567

[8] Windaus, A , *Ber chem Ges* , 1909, xlii, 244

0 1320 gm of substance (dried at 110°C) gave 0 2998 gm of CO_2 and 0 1034 gm. of H_2O

Found		Calculated ($C_{55}H_{142}O_{30}$)	
C	H	C	H
per cent	per cent	per cent	per cent
61 94	8 70	62 119	8 64

According to Hausmann and Abderhalden, a sterol, containing an hydroxyl group and a double-bond in its molecule, is capable of preventing hemolysis by saponin, and when the hydroxyl group is replaced by another atom or group, or linked with such an atom or group, as is the case in an ether, its action disappears. If the double-bond is saturated, its action becomes weaker than that of the initial material. Referring to this conclusion, mycosterol probably contains a free hydroxyl group, but does not contain a double-bond. To test these assumptions the following experiments were carried out.

Acetylation.—In a small flask fitted with a condenser, 0.5 gm. of mycosterol and twenty times its volume of acetic anhydride were boiled gently on a sand bath for 5 hours. After standing, the crystals which precipitated out were filtered off, washed with acetic acid, and recrystallized from ether, melting at 169°C.

0 1524 gm. of substance gave 0 4448 gm. of CO_2 and 0 1504 gm. of H_2O

Found		Calculated ($C_{32}H_{50}O_3$)	
C	H	C	H
per cent	per cent	per cent	per cent
79 59	10 96	79 66	10 37

Bromination.—0.123 gm. of mycosterol was dissolved in 5 cc. of ether and to this solution a bromacetic acid mixture was added. The ether was then allowed to evaporate spontaneously, leaving colorless needle-shaped crystals. The melting point, color reaction, and solubilities agreed with those of mycosterol; therefore, the crystals may be considered to be the unchanged mycosterol.

It is evident from the above experiments that mycosterol contains at least one free hydroxyl group in its molecule, but does not contain a double-bond which can be tested by the methods above mentioned.

With regard to the manner in which the other atom of oxygen is linked up in the molecule of mycosterol, there are several possibilities. Reasoning from the oxidation products of cholesterol, however, the carbonyl group or hydroxyl group

$$C_{29}H_{47}{\Big<}{}^{CO}_{OH} \quad or \quad C_{30}H_{46}{\Big<}{}^{OH}_{OH}$$
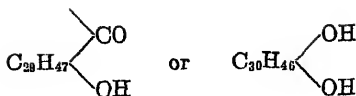
is most probably to be taken into account. Since mycosterol, when treated with phenylhydrazine, does not form a phenylhydrazone, it may be concluded that it does not contain the carbonyl group for which this is a test. If the oxygen may exist as a carbonyl group it should be in a state which cannot be readily acted upon by phenylhydrazine, as oxycholesterol, obtained by Mauthner[9] oxidizing cholesterol, does not readily form a phenylhydrazone, though containing the carbonyl group.

0.5 gm. of phenylhydrazine hydrochloride and 0 5 gm of sodium acetate were added to 1 gm of mycosterol dissolved in warm alcohol. The mixture was heated for 1 hour on a water bath under a reflux condenser   Upon cooling crystals precipitated, which were filtered off by means of suction, washed with alcohol, and recrystallized from warm alcohol   The crystal form and the melting point were in agreement with those of mycosterol, and as the compound contains no nitrogen, it must be mycosterol itself

Oxidation of Mycosterol.—As has already been shown, mycosterol has one hydroxyl group, which is verified by the action of acetic anhydride. Therefore, if the other oxygen exists as a hydroxyl group, it must be in a form which cannot be detected by acetylation, as is often the case with a tertiary alcohol group [10] The decision between the two possibilities can be made by examining the oxidation products of mycosterol. On treatment of mycosterol with chromic acid it yielded three different neutral crystalline products and an analysis of the substance of which the largest amount was obtained agreed with the compound $C_{30}H_{48}O_3$

5 gm. of chromic acid dissolved in 10 cc. of acetic acid were carefully added to 10 gm ' of mycosterol which were suspended in 100 cc of acetic acid. The mixture was heated on a water bath until it was deep green   The resultant solution was poured into a diluted sodium chloride solution, the whole then extracted with ether. The ether layer was treated with sodium chloride solution, then with sodium hydroxide solution to remove acid substances.

[9] Mauthner, J , and Suida, W , *Monatsh Chem* , 1896, xvii, 582
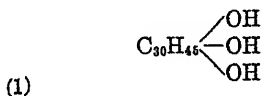[10] Diels, O , and Abderhalden, E , *Ber chem Ges* , 1903, xxxvi, 3178

The oily residue mixed with crystals formed on evaporation was washed with petroleum ether and benzene and recrystallized from acetone, melting at 188–189°C.

This substance is soluble in acetone, alcohol, and chloroform, and insoluble in water.

0 118 gm of substance (dried at 105°C) gave 0 3404 gm of CO_2 and 0 1168 gm of H_2O

Found		Calculated ($C_{30}H_{48}O_3$)	
C *per cent*	H *per cent*	C *per cent*	H *per cent*
78 67	10.99	78 94	10 53

According to these results the oxidation product may be regarded as an additional product of one atom of oxygen and one molecule of mycosterol, for the number of carbon and hydrogen atoms remains equal to that in mycosterol.   Considering the relation of the new oxygen toward the molecule, two possibilities may be suggested, namely, either that one of the groups containing oxygen in the molecule of mycosterol has been converted into a carbonyl group by the addition of one oxygen, or that a new group containing oxygen has been formed.

$$C_{30}H_{46}{\Large\langle}\begin{matrix}OH\\OH\\OH\end{matrix} \qquad C_{29}H_{46}{\Large\langle}\begin{matrix}COOH\\OH\end{matrix}$$

(1)   (2)
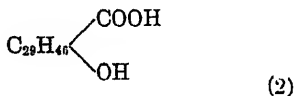
Since the oxidation products are neutral, the former case is out of the question, and since the number of hydrogen atoms in the molecule of the new substance is equal to that of the original mycosterol, the hydroxyl group and the hydrocarbon group must have remained untouched.

It is, therefore, reasonable to conclude that in the molecule of the oxidation product two out of three groups containing oxygen remain the same as in mycosterol, while the third is a newly formed hydroxyl group.   To prove this point 1 gm. of the oxidation product was mixed with twenty times its volume of acetic anhydride and boiled gently on a sand bath for 5 hours Upon cooling, water was added until the solution became a little turbid.   Long needle-shaped crystals were precipitated which were washed thoroughly with a little ligroin and recrystallized from acetic acid, melting point 201–202°C

0 1647 gm. of substance (dried at 105°C) gave 0 4454 gm of CO_2 and 0.141 gm of H_2O

	Found		Calculated ($C_{36}H_{64}O_6$)	
C		H	C	H
per cent		*per cent*	*per cent*	*per cent*
73 92		9 3	74 22	9 27

The substance which was obtained by treating the oxidation product with acetic anhydride is a triacetic ester as is shown in formula (1).

From this fact it may be concluded that two atoms of oxygen in mycosterol are contained as hydroxyl groups and mycosterol may be a dihydroxyl compound and its oxidation product a trihydroxyl compound.

$$C_{30}H_{46} \begin{cases} OH \\ OH \end{cases} \qquad C_{30}H_{45} \begin{cases} OH \\ OH \\ OH \end{cases}$$

Mycosterol   Oxidation product.
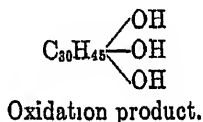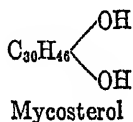
ON THE RELATIVE ACCURACY OF COLORIMETRIC AND TITRIMETRIC PROCEDURES FOR DETERMINING NITROGEN AS AMMONIA.

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INTRODUCTION

The problems arising in connection with investigations in the field of soil biology frequently are concerned with the fixation and transformation of nitrogen and require nitrogen determinations made with greater accuracy than is the case in most other lines of work. While engaged in this field of research, we carried out a considerable number of studies on nitrogen methods. Among other points studied, the comparative accuracy of the colorimetric and titrimetric procedures for the determination of nitrogen in the ammonium form have been investigated

In the matter of improvement and adaptation of nitrogen methods to particular fields of research, two workers, Folin in America and Mitscherlich in Germany, have been particularly active. Folin, working in the field of animal nutrition, has employed both colorimetric and titrimetric procedures and favors the former, whereas Mitscherlich, studying the problems of plant nutrition, has adhered to the titrimetric procedure Folin and Farmer[1] used a modified Nessler-Winkler reagent in such a way that as much as 1 5 mg of nitrogen may be determined colorimetrically without precipitation. By proper aliquoting, the quantity of nitrogen is kept below this amount

Methods of this type have found quite wide favor, and certain modifications have been proposed from time to time by Folin and

[1] Folin, O., and Farmer, C. J., *J. Biol Chem* , 1912, xi, 493

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his associates   On the other hand Mitscherlich and Herz[2] used titration with ± 0 02 N solutions with a surprising degree of accuracy for amounts of nitrogen varying from 0 68 to 6.84 mg.   However, it was not clear from the papers of these two workers which procedure was the more accurate, and therefore the work reported below on the probable error of the two procedures, as computed by the method of least squares, was carried out

EXPERIMENTAL

Reagents and Solutions.

Indicator Used —The compound dimethylaminoazobenzene-*o*-carboxylic acid, or methyl red, is the most suitable indicator for the titration of weak bases as ammonia, and is extensively used at the present time.   It has rightly supplanted the use of alizarine red, Congo red, cochineal, and lacmoid   Its end-point is so sharp, even in the presence of ammonia, that if proper conditions are observed very dilute solutions are titrated with ease and accuracy.

Titrimetric Standards —Standard 0 02 N acid was prepared from pure sulfuric acid and carbon dioxide-free water, and standardized by the sodium carbonate method, which, according to Mitscherlich and Meeres,[3] is the most accurate.   Standard 0 02 N alkali was prepared from sodium hydroxide and carbon dioxide-free water[4]   The titrations were made with the aid of 25 cc burettes which were of regular 50 cc. length, but were correspondingly smaller in internal diameter, and were graduated to 0 05 cc.[5] These burettes were calibrated by the Bureau of Standards for 20°C., and that temperature was maintained as nearly as possible

Analytical Standards —Standard solutions of ammonia were carefully prepared from ammonium hydroxide and from ammonium sulfate   The former were prepared from concentrated c. p. ammonium hydroxide and neutral[6] distilled water, and the

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[2] Mitscherlich, E A , and Herz, P , *Landw Jahrb* , 1909, xxxviii, 279

[3] Mitscherlich, E A , and Meeres, E , *Landw Jahrb* , 1910, xxxix, 345

[4] We have subsequently found that stock distilled water redistilled over acid permanganate is sufficiently pure for the preparation of standard solutions

[5] These were obtained from Emil Greiner and Company, New York

[6] The term "neutral water" as used in this paper refers to water neutral to methyl red

exact strength determined by a series of titrations against the titrimetric standards. The ammonium sulfate standards were prepared from ammonium sulfate, repurified according to the directions of Folin

Nessler Reagent.—This reagent was prepared according to the directions of the Standard Methods of the American Public Health Association [7]

Nessler-Winkler Reagent —This reagent was prepared according to the original direction of Winkler[8] and used according to Folin and Farmer,[9] who made the observation that if the reagent is diluted just before use (5 cc. to 25 cc.) larger amounts of ammonia may be Nesslerized without precipitation

Colorimeter.—A study of the Kruss polarization, and Schreiner, Wolff, and Duboscq colorimeters led to the adoption of the last named as being the most suitable for colorimetric work The instrument used was of the standard French pattern.

Probable Error of Nesslerization

In Nesslerization the error lies principally in the difference in the amount of color produced by the action of the reagent on the ammonia and in matching the unknown against the standard

[7] Methods of water analysis of the American Public Health Association, Standard methods for the examination of water and sewage, Boston, 3rd edition, 1917, 16   50 gm of potassium iodide in a minimum quantity of cold water were treated with saturated solution of mercuric iodide until a slight precipitate persists permanently   Add 400 cc of 50 per cent potassium hydroxide, clarified by sedimentation, and dilute to 1 liter, allow to settle, and decant

[8] Winkler, L W , *Chem Z* , 1899, xxiii, 541   Hawk, P B , Practical physiological chemistry, Philadelphia, 6th edition, 1918, 517

"Mercuric iodide	.	10 gm
Potassium "		5 "
Sodium hydroxide		20 "
Water		100 cc

The mercuric iodide is rubbed up in a small mortar with water, worked into a flask, and the potassium iodide added   The sodium hydroxide is dissolved in the remaining water, and the cooled solution is added to the above solution   After settling and decanting, the solution is kept in a dark bottle "

[9] Folin, O , and Farmer, C J , *J Biol Chem* , 1912, xi, 496

The former error can be reduced to a minimum by observing all possible precautions to keep conditions uniform, such as the amount of reagent, temperature, time of standing before comparison, etc., while the latter error may be decreased by increasing the number of readings. In all colorimetric work reported in this paper, six readings were made on each unknown and its standards. The error arising from matching of unequal column heights in the colorimeter was eliminated by preparing the "standard" and "unknown" of practically equal concentrations.

The standard Nessler reagent was retained for use with amounts of nitrogen of 0 5 mg. per 100 cc.[10] or less, since it is more sensitive, and in the presence of small amounts of nitrogen more stable than is the modification of Winkler.[11]

The Nessler-Winkler reagent was used according to the directions of Folin and Farmer.

By using these reagents with the above amounts of nitrogen and employing only redistilled water, there was not the slightest tendency towards the formation of cloudiness The colors remained perfectly clear and manifested no tendency to precipitate even after several hours The use of ordinary distilled water, freed from ammonia by bromine and caustic soda, for diluting the reagent and making the Nesslerized solution to the mark, as has been recommended by Folin and Denis,[12] did not prove satisfactory, as there was some tendency for cloudiness to form on the addition of the reagent

For amounts of ammonia nitrogen of 0.5 mg and less, using the standard Nessler reagent, the procedure was as follows: The

[10] The expressions "mg N" and "mg N per 100 cc " used in this paper in connection with Nesslerization refer to mg of nitrogen contained in the colorimeter solution, i e , the solution after the addition of the Nessler reagent and dilution to the mark

[11] This is probably due to the difference in the proportions of the mercuric salts and the potassium iodide in the two reagents   The sensitiveness increases with addition of mercuric salts and decreases by the addition of potassium iodide (see Olsen, J C , Quantitative chemical analysis, New York, 5th edition, 1916, 412)   Folin and Denis (Folin, O , and Denis, W , J Biol. Chem , 1916, xxvi, 479) point out, however, that for satisfactory results with their modified reagent, it should contain more potassium iodide than does the standard Nessler's reagent

[12] Folin, O , and Denis, W , J Biol Chem., 1912, xi, 534

desired amounts of ammonia were measured into 100 cc graduated flasks from the ammonium hydroxide or ammonium sulfate standards by means of burettes. These solutions were then diluted to approximately 90 cc with ammonia-free water, 2 cc of Nessler's reagent were added, and the solutions were made to the mark. After 15 minutes, portions of the solutions were transferred to the colorimeter cylinders, and the colors compared, as mentioned above. The standard was set at 40 mm and three of the six readings were made after adjusting the unknown from above, and three after adjusting from below.

For amounts of nitrogen equal to 0 5 mg. or more per 100 cc requiring the Nessler-Winkler reagent, the procedure is the same except that the solutions are made to 70 cc. in the flasks, 25 cc of the diluted reagent added; they are then made to the mark, and the colorimetric comparisons made against a 20 mm. standard 30 minutes after adding the reagent. Six readings were made as described above

The desired amounts of ammonia nitrogen were obtained by using the following amounts of dilute solutions.

N_2	Solution	Normality
mg	cc	
0 10	10 0	N/1,400
0 50	50 0	N/1,400
1 00	10 0	N/140

For each amount of nitrogen, ten solutions (also ten standards[13]) were Nesslerized. Although the probable error of a method should be obtained by employing the results from a great many determinations using the procedure involved, the *order of magnitude* of the error of the procedure can be shown by using ten typical determinations of the procedure studied.

The probable error, r, of each individual observation is calculated with the aid of the formula

$$r = \pm 0\,6745\,\sqrt{\frac{\Sigma\,\Delta^2}{n-1}}$$

in which Δ indicates the deviation of each value from the mean, and n the number of observations. The results are shown in Table I.

[13] The ammonium sulfate solutions were taken as "standards," the hydroxide solutions as "unknowns"

The results show that the probable error increases with increasing amounts of nitrogen   This must unavoidably be the case since with the larger amounts of nitrogen the colors produced are intense, a low column height ($\pm$ 20 mm.) must be used in the colorimeter, and very slight differences in readings result in marked differences in the value found for mg. of nitrogen.

Computations were next made to ascertain to what extent the errors in the colorimetric procedure as used lay in the readings themselves and to what extent in the color produced by the reagent

<div align="center">TABLE I</div>
<div align="center">Probable Error of Nesslerization</div>

Determination No	Nitrogen taken		
	mg 0 10	mg 0 50	mg 1 00
	Found		
1	0 106	0 468	0 972
2	0 098	0 516	0 996
3	0 102	0 511	1 014
4	0 102	0.497	1 013
5	0 103	0 508	1 004
6	0 102	0 489	0˙ 995
7	0 104	0 507	1 008
8	0 105	0 476	0 962
9	0 108	0 504	0 995
10	0 106	0 487	1 002
Average	0 103	0 496	0 996
`	$\pm$0 0019	$\pm$0 0107	$\pm$0 0114
` expressed as per cent	1 8	2 1	1 1

As stated above, each colorimetric determination is the average of six readings, and of course possesses an error, that of reading the colorimeter.   That is, if we designate the value obtained by the colorimetric determination by a and its error by e the correct value is equal to $a \pm e$.   In a series of determinations, we designate these values by $a_1 \pm e_1, a_2 \pm e_2, a_3 \pm e_3 \quad a_n \pm e_n$.   Where the values of a_1, a_2, a_3 are obtained by averaging a number of readings as was done in this case, the values of e_1, e_2, e_3, i.e. the probable error R_1, R_2, R_3 of the average, may be computed with the aid of the formula

$$R = \pm 0\,6745 \sqrt{\frac{\Sigma \Delta^2}{n(n-1)}}$$

The values of R_1 to R_5 (e_1 to e_5) were computed for a_1 to a_5 of the determinations of 1 mg. portions, reported in Table I   The results appear in Table II

Since the values obtained for R_1 to R_5 are in every case less than the value ± 0.0114 obtained for the probable error of a set of determinations, it follows that the color produced by the same amount of nitrogen is not perfectly concordant in a series of determinations

The error in the colorimetric method for nitrogen then increases with increasing amounts of nitrogen, and is due in part to error in

TABLE II

Probable Error of Arithmetical Mean of Colorimeter Readings on the Same Solution

Reading No	Determination No				
	1	2	3	4	5
1	0 982	1 006	1 006	1 001	1 011
2	0 965	0 996	1 015	1 021	1 011
3	0 961	0 988	1 011	1 015	1 006
4	0 982	1 001	1 025	1 025	0 996
5	0 961	0 992	1 011	1 001	1 006
6	0 978	0 992	1 015	1 015	0 996

a_1 0 972, a_2 0 996, a_3 1 014, a_4 1 013, a_5 1.004
R_1 $\pm 0\,0028$, R_2 $\pm 0\,0018$, R_3 $\pm 0\,0016$, R_4 $\pm 0\,0028$, R_5 $\pm 0\,0019$

measurement, and in part to differences in the amounts of color produced by the reagent under the conditions of the determinations

The Probable Error of Titration

In order to avoid all error due to the solubility of the glassware commonly employed as titrimetric flasks, well seasoned Pyrex glass Erlenmeyer (300 cc) flasks were used in determining the probable error of titration.

Standard ammonium hydroxide was run into the titration flasks from a burette, 25 cc of 0 02 N acid were added, then sufficient neutral water was added to bring the volume to 100 cc , the con-

tents were boiled to expel carbon dioxide, and after cooling to
10–15° the titration was completed, using 0.02 N alkali. The
error reported here includes the error of the burettes, but since
this is less than that tolerated by the Bureau of Standards, the
errors reported in Table III are essentially attributable to the
titration alone.

TABLE III

Probable Error of Ammonia Determination by Titration

Nitrogen taken		
mg 0 100	mg 0 500	mg 1 00
Found		
0 101	0 502	1 001
0 098	0 505	1 002
0 093	0 505	0 995
0 098	0 505	1 001
0 093	0 510	0 992
0 099	0 508	1 001
0 093	0 499	0 992
0 101	0 508	1 005
0 098	0 502	1 005
0 093	0 502	1 006
Average 0 097	0 505	1 000
r ±0 0022	±0 0022	±0 0035
r expressed as per cent. 2 06	0 38	0 35

The results of the two methods of ammonia determinations as
summarized in Table IV show some interesting differences.

TABLE IV

Nesslerization			Titration	
N₂ taken	Probable error	Error	Probable error	Error.
mg	mg	per cent	mg	per cent
0 10	±0 0019	1 80	±0 0022	2 06
0 50	±0 0107	2 10	±0 0022	0 38
1 00	±0 0114	1 10	±0 0035	0 35

The probable error of Nesslerization is variable and tends to
increase with increasing amounts of ammonia while that of

-titration remains practically constant. For quantities of nitrogen above 0.50 mg portions, titration must be considered more accurate than Nesslerization. With considerably less than 0.50 mg. portions of ammonia nitrogen, Nesslerization is slightly more accurate than titration.

The Probable Error of Distillation and Titration.

It sometimes happens that the ammonia in an unknown may be determined directly by Nesslerization, whereas in the case of the titration procedure, the ammonia must always be transferred by distillation (or aeration) to a measured amount of standard acid.   Indeed Folin and Denis,[14] and Folin and Bell[15] have recently proposed a series of very convenient methods in which the nitrogen is determined directly by Nesslerization.   Thus the question arises as to the error of titration plus the error of distillation.   Accordingly the following series of determinations was carried out.

The distilling apparatus used in estimating the probable error of distillation and titration has been previously described.[16]   This apparatus is of Pyrex glass with the exception of one close rubber connection which was found not to vitiate the results when working with very small amounts of nitrogen   While this type of apparatus is not recommended for general analytical work, it has been found excellent for the most careful work where small differences must be determined.   However, by using the devices described elsewhere,[17] together with good block tin condensers, and the modified Benedict procedure of distillation[18] very accurate results can be obtained with small amounts of nitrogen.   The results obtained on 1 mg. of nitrogen are reported in Table V

The probable error of distillation and titration is of the same order of magnitude as the probable error of titration alone.   In other words the error of titration and distillation on a 1 mg. portion

[14] Folin, O , and Denis, W , *J Biol Chem* , 1916, xxvi, 473, 491, 497, 501, 505

[15] Folin, O , and Bell, R D , *J Biol Chem* , 1917, xxix, 329

[16] Allen, E R , and Davisson, B S , *Ann Missouri Bot Gardens*, 1919, vi, 45

[17] Davisson, B S , *J Ind and Eng Chem* , 1919, xi, 465

[18] Allen, E R , *Ann Missouri Bot Gardens*, 1919, vi, 23

is less than that accompanying direct Nesslerization of an equal amount

Although no data are included here for larger amounts of nitrogen, the probable error remains almost constant in absolute value and decreases in percentage with larger amounts of nitrogen. Considerable data on more complicated apparatus and with larger amounts of ammonia nitrogen showed but little greater error.[19]

TABLE V

Probable Error of Distillation and Titration

Nitrogen taken		
$\frac{mg}{1\ 00}$		
Found		
	1 005	
	0 996	
	1 010	
*	0 998	
	1 013	
	1 002	
	0 996	
	1 007	
	1 005	
	1 005	
Average .	1 0037	
r	±0 0038	

Preparation, Preservation, and Use of 0 02 N Solutions.

Since more precaution must be taken in the use of 0.02 N solutions than is usually observed in volumetric analysis, it seems worth while to record at this point the procedures which, after extended trial, have proved satisfactory.

As an acidimetric standard we have found sodium carbonate satisfactory from the standpoint of convenience and accuracy   It has the advantage that the standardization is carried out under conditions practically the same as are encountered in the regular work.

[19] Allen, E R , *J Ind and Eng Chem* , 1915, vii, 521   Davisson, B S., Allen, E R , and Stubblefield, B M , *ibid* , 1916, viii, 896   Davisson, B S , *ibid* , 1918, x, 600, 1919, xi, 465

The sodium carbonate is prepared from pure sodium bicarbonate by heating to 270–300° in platinum until constant weight is obtained. The bicarbonate is easily prepared in a high degree of purity, either by recrystallizing of a high grade product,[20] or by carbonating a filtered concentrated solution of normal carbonate.

It has been our practice never to prepare solutions *exactly* 0.02 N. The volumetric adjustment of large volumes of solutions is not entirely trustworthy unless the final solution is restandardized. Where logarithmic computations are used nothing is to be gained by having the solutions of an exact fraction of normality unless perhaps when they are used for a variety of purposes. Moreover, in the process of standardization no attempt is made to weigh out an exact amount of a molar weight of sodium carbonate, since such a procedure is inconvenient and, because of the prolonged exposure of the powdered material to the air, is likely to be inaccurate.

20 or 25 cc. portions of approximately 0.02 N acid are titrated each time. The sodium carbonate equivalent to such quantities of solutions is a rather small amount to weigh with extreme accuracy This difficulty is, however, readily avoided by the method of aliquoting which has been recommended by Eastlack.[21] The strength of the acid is expressed as its *nitrogen titer; i e.,* its *nitrogen equivalent in gm. (or mg.) per cc* The computation is easily made with the aid of logarithms.

It has been our practice to prepare 8 to 10 liter portions of solutions at one time. The reservoir bottles are provided with soda-lime guard tubes and the solutions are supplied to the burettes preferably through siphon tubes of Pyrex glass The acid solution does not change strength appreciably on standing In Table VI are given the results of change in concentration of a solution of approximately 0 02 N H_2SO_4. The original volume was 8 liters

The sodium carbonate was in all cases prepared from Kahlbaum's bicarbonate "Zur Analyse." In Standardization I the bicarbonate was recrystallized, while in the subsequent standardizations this step was omitted The variations in the first four determinations are insignificant. The higher value reported in

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[20] Olsen, J C , Text-book of quantitative chemical analysis, New York, 5th edition, 1916, 259

[21] Eastlack, H E , J Am Chem Soc , 1918, xl, 620

the last standardization may have been due to the fact that when the standardization was made, less than 1 liter remained in the bottle, and the walls of the bottle were covered with water which had vaporized and condensed.[22] The maximum variation, however, between the lowest and highest value in the above standardizations amounts to only 0 0018 mg. of nitrogen per 1 cc. or 0 045 mg per 25 cc. of acid, an error therefore of less than 1 per cent, and which may be disregarded except in the most exact work. Another large bottle of acid, which had been in use for a little over 11 months, showed a nitrogen titer of 0.2835 mg. at the beginning and 0.2850 at the end of this period.

The question of the deterioration of $\pm$ 0.02 N alkali solution was also studied. In Table VII are given the results of the determinations at different intervals.

TABLE VI.

Standardizations of Approximately 0 02 N H_2SO_4

Standardization No	Date	Nitrogen titer
I	Nov 19, 1917	0 2825
II	" 13, "	0 2827
III	Dec 17, "	0.2833
IV	Jan. 23, 1918	0 2833
V	Mar 26, "	0 2843

From this it is seen that the change in strength of the alkali solution in 1 month is quite appreciable. It has become our practice to check the alkali solution every week. No attempt has been made to prepare or keep the alkali exactly equivalent to the acid since the cc of alkali obtained in any titration is readily converted into cc of alkali exactly equivalent to the acid with the aid of the equation.

$$\text{Log } c = \log A - \log \frac{\text{cc of alkali}}{\text{cc. of acid}} \left(\text{or} + \log \frac{\text{cc. of acid}}{\text{cc of alkali}} \right)$$

where c = cc of alkali of exactly the same strength as the acid and A = the observed cc of alkali. The ratio $\left(\dfrac{\text{cc of alkali}}{\text{cc of acid}} \right)$ is, of course, determined in previous titrations

[22] It has subsequently been found that a layer of white paraffin oil on the surfaces of the solutions prevents loss from evaporation

In the use of 0.02 N solutions certain precautions are naturally necessary which may be disregarded in the more concentrated solutions.   We have found that neglecting any of the following measures introduces an appreciable error

1 The volume of solution in the titrating flasks should be kept small and constant (*i.e.* within 100 to 125 cc.), and should be well cooled and free from carbon dioxide.   This procedure reduces the error from the hydrolysis of the indicator salt to a small and constant magnitude, and insures a sharp and certain end-point.

2. An approximately constant amount of standard acid should be used in each titration in order to avoid any "salt effect" on the indicator.   Thus, if the ratio between the acid and the alkali is established on 25 cc. portions and 50 cc of acid are used in a determination, a slight error is introduced

TABLE VII

Change in Strength of $\doteq$ 0 02 N Alkali

Determination No	Date	Alkali equivalent to 25 cc. of acid
I	Nov 9, 1917	25 72
II	Dec 14, "	25 55
III	Jan 16, 1918	25 47
IV	Feb 14, "	25 34
V	Mar 20, "	25 16

3. Standard solutions must be properly prepared and their strength determined in the dilution in which they are finally used.

4. Solutions which have stood 12 hours or more in the burettes must be discarded

5. The titration flasks must be of practically insoluble glass. Pyrex glass is the only material (except transparent quartz) which we have found to be entirely satisfactory.

DISCUSSION.

From the results reported above, which represent it is believed a fair measure of the relative accuracies of the titrimetric and colorimetric methods for determining ammonia, it seems that the latter are slightly more accurate.   Both types of determinations have been made, it is true, under conditions more nearly ideal than

one can attain in general practice, yet the conditions described for accurate titrations may be more nearly attained than can those for satisfactory Nesslerization. Titrimetric procedures have the added advantage over the colorimetric in that they are applicable to a wide range of nitrogen values, and hence by a regulation of the size of the sample the error due to aliquoting may be kept smaller. For example, if we have a quantity of material for analysis containing 10 gm. of nitrogen, $\frac{1}{10,000}$ of the material would be taken for the determination by the colorimetric procedure. If our readings show 1 00 mg., the value is obviously 1 00 $\pm$ 0 0114 mg., and the amount of nitrogen in the material sampled would be between the limits of 10 114 and 9.886 gm. Only the first digit to the right of the decimal would be of any significance   On the other hand, if the titrimetric method is used, $\frac{1}{2,000}$ of the material may be taken for analysis   If our determination shows a value of 5.000 mg., the true value is 5.000 $\pm$ 0 0038 mg., and our final value lies between the limits 10 008 and 9 992, in which case the first digit to the right of the decimal is significant, the second doubtful, and the third of no significance   Expressed in another way the error in the colorimetric and titrimetric procedures would be under the best conditions 0 228 and 0 015 gm. respectively

The disadvantage of the colorimetric determination of ammonium nitrogen is that the reaction involved is imperfectly understood, and it is not surprising, therefore, that in spite of the different modifications of the Nessler reagent which have appeared, the appearance of clouds and precipitates cannot be avoided with certainty by different workers.

Aside from this point, it is our opinion that the colorimetric methods are, even under the best conditions, more subject to personal error than are volumetric or titrimetric procedures. The accurate measurement of color is a more elusive and difficult operation than is generally believed and many colorimetric procedures are quite faulty in theory.

Some of the more common sources of error will be briefly considered. One which may become quite appreciable in routine colorimetric work (but which was avoided in the determinations reported above) is that due to unequal column heights, especially if the procedure of setting the standard at a definite height and

adjusting the unknown is too rigidly followed. The fact must not be lost sight of that the ordinary equation of colorimetry

$$c_1 = c_2 \frac{h_2}{h_1}$$

is based on the incorrect assumption that the solvent has no effect on the passage of light   If this error is reduced to a negligible magnitude either by the preparation of a large number of standards or by the scrupulous filtering of all solutions. as has been recommended by Kruss and Kruss[23] for colorimetric procedures in general, then the procedure becomes distinctly time-consuming.

It is of interest to note in this connection that Dehn[24] in a recent paper on "Fallacies in colorimetry," after pointing out the unsoundness of many existing procedures, concludes that in Folin's method of creatinine estimation, by way of illustration, there are twelve probable sources of error   A similar array also exists in the colorimetric determination of ammonium nitrogen. These fundamental errors of colorimetry probably account for the skepticism with which many chemists are inclined to view colorimetric procedures.

In view of these points we have therefore adhered to the titrimetric procedures in our work on nitrogen methods.

While the colorimetric procedures for the determination of nitrogen have been of great service because of their brevity, it should be recognized that except when dealing with amounts of nitrogen less than a few tenths mg , titrimetric methods are capable of greater accuracy and should be chosen where exact results are required.

[23] Kruss, G , and Kruss, H , Kolorimetrie und quantitative Spektral-analyse in ihrer Anwendung in der Chemie, Hamburg, 1909, 31
[24] Dehn, W. H , J Am Chem Soc , 1917, xxxix, 1392, 1399

A STUDY OF CREATINURIA IN INFANTS.

I. RELATION OF CREATINURIA TO ACIDOSIS. THE ELIMINATION OF INGESTED CREATINE AND CREATININE.

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Creatine apparently never appears in the urine of a normal adult male in appreciable amounts.   Normal women[1] excrete creatine periodically. while infants and children[2] exhibit a creatinuria regularly on normal diets.   Creatine metabolism must, therefore, be studied in both sexes, and at different stages of growth, if a complete set of facts regarding the processes concerned is to be obtained.   We have undertaken to put to experimental test in the male infant certain factors supposed to bear relation to creatinuria.   We have also made measurements of the elimination of ingested creatine and creatinine by infants, there being no quantitative data at hand on this point at this age

Methods.

The subjects used for the experiments described in this and the succeeding paper were infants obtained from a well conducted home for foundlings.   One of them, Subject G, was a normal infant.   The others failed to come up to the specifications for normal infants only in the respect that they were more or less underweight.   There was in no instance a history of recent nutritional disturbance, or, during the experimental periods, symptoms

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[1] Krause, R A , *Quart J Exp Physiol* , 1911, iv, 293   Rose, M S , *J Biol Chem* , 1917, xxxii, 1

[2] Rose, W C , *J. Biol Chem* , 1911–12, x, 265   Folin, O., and Denis, W , *ibid* , 1912, xi, 253

of malnutrition. They all made moderate or rapid gains in weight on the experimental diets. The diet was in all cases cow's milk, the various modifications of which, as regards the protein, butter fat, and lactose content, are indicated in Tables I and II. The quantity of food given was carefully measured and all of it was taken. There was always a foreperiod of several days on the experimental diet before the collection of urine specimens was begun.

The urine collection periods were of 2 or more days duration. The infant during this time was immobilized by means of a comfortably arranged metabolism frame. The urine was preserved with 5 per cent thymol in chloroform. The bottle receiving the urine was stoppered and shaken every 4 hours to insure thorough disinfection, and at the end of 12 hours was replaced by a second bottle and put in an ice box. The regularly close agreement of the day to day figures indicates the quantitatve collections obtained.

Creatinine and creatine were determined by the methods of Folin.[3] Conversion of creatine was obtained by autoclaving the urine samples, 5 to 10 cc., at 130° for 18 to 20 minutes with 1 cc. of N hydrochloric acid. The acid was neutralized by adding an equivalent of 10 per cent sodium hydroxide before developing the color. Total nitrogen in milk and urine was determined by the Kjeldahl method. The factors of acid excretion in the urine, hydrogen ion concentration, titratable acidity, and ammonia, were measured as directed by Palmer and Henderson.[4]

Relation of Acidosis to Creatinuria.

One gathers from the literature the impression that acidosis stands accepted as a cause of creatinuria, or, at least, as a factor in its production. It is our belief that the presence of an actual acidosis has been in most instances only a matter of inference, and, furthermore, that in conditions where acidosis and creatinuria undoubtedly do occur together evidence of a causal relationship is lacking.

[3] Folin, O , J Biol Chem , 1914, xvii, 469
[4] Palmer, W W., and Henderson, L J , Arch Int Med , 1913, xii, 153.

Krause and Cramer,[5] in 1910, pointed out that creatinuria occurs in all conditions which lead to an acidosis. They cited chiefly the creatinuria of starvation and of diabetes. At that time, however, the fallacy of regularly inferring an acidosis from the presence in the urine of ketone acids was prevalent. Since we know now that production of ketone acids is only occasionally of such degree as to cause an acidosis it is obviously incorrect to regard the occurrence together of ketonuria and creatinuria as proof of the relation of the latter to acidosis.

Underhill[6] has published results of experiments with rabbits, designed to show a relation between creatinuria and diets which produce an acid urine. An acid urine was produced by giving the rabbits a grain diet composed of corn and oats. On this diet, creatine was regularly found in the urine. When carrots were given, instead of the grain diet, creatine was found in the urine in greatly diminished quantity, or was absent   Underhill considered these results as proof of a relationship between creatinuria and acidosis. The presence of acidosis "was considered indicated when the urine of the rabbit became strongly acid, as shown by determination of the hydrogen ion concentration   . . .   In the sense of the term acidosis as used here, that is, a condition of alkali depletion, the assumption given above is undoubtedly correct since ordinarily the rabbit secretes urine which is strongly alkaline." Since rabbits cannot provide the base ammonia in compensation for an unusual acid production, it is probable, though not definitely proven by the acid urine, that the rabbits Underhill experimented with did develop an acidosis.

From the well established facts in regard to the process of acid excretion in man, it is absolutely incorrect to assume a depletion of fixed alkali from an unusually acid urine. The neutrality mechanism in man is remarkably extensible and is capable of neutralizing and conveying into the urine unusually large amounts of acid without disturbance of the acid-base equilibrium within the body. The gross adjustment for an unusual acid production is an increase in production of ammonia. The fine adjustment, by means of which the reaction of the blood is maintained at the

[5] Krause, R A , and Cramer, W , J Physiol , 1910, xl, p. lxi
[6] Underhill, F P , J Biol Chem , 1916, xxvii, 127, 141.

TABLE I

Effect of Ingested Alkali and Acid upon Output of Urinary Creatine.

Subject G, age 10 months, weight 6 5 to 7 1 kg.

Experiment No	Date	Protein per day	Volume	Urine								Remarks
				Total N	pH	Titratable acid 0.1N	NH3 0.1N	Total acid 0.1N	Total creatinine	Preformed creatinine	Creatinine as creatinine	Diet
		gm	cc	gm		cc	cc	cc	mg	mg	mg	
1 Control period	1917 Mar 17	20	500		6 4	44	125	169		78		Milk, 600 cc
	" 18	20	550		6 4	41	130	171		76		Barley water, 300 cc
	" 19	20	500		6 4	36	121	157		77		Lactose, 40 gm
	" 20	20	490		6 5	32	118	150		79		
	Averages	20	510	1 89	6 4	38	124	162	137	78	59	
Experimental period	Mar 21	20	500		8 0	−43	32		137	82	55	Same food + 4 gm of NaHCO3
	" 22	20	490		8 2	−39	40		133	82	51	Same food + 4 gm of NaHCO3
	" 23	20	540		8 1	−40	40		137	81	57	Same food + 4 gm of NaHCO3
	" 24	20	470		8 3	−40	35		135	84	51	Same food + '4 gm of NaHCO3
	Averages	20	500	1 97	8 2	−41	37	−4	136	82	54	
2 Control period.	May 7	30	490		6 2	100	240	340	187	82	105	Whole milk, 900 cc
	" 8	30	490		6 4	90	204	294	175	81	94	Lactose, 20 gm
	Averages	30	490	4 37	6 3	95	222	317	182	82	100	

Period	Date											Notes
Experimental period	May 10	30	570		7 6	−100	92		191	83	109	Began NaHCO₃, 36 hrs. before and continued through May 10 4 gm per day.
	" 11	30	520		7 6	−25	92		190	82	108	No NaHCO₃ given
	Averages	30	545		7 6	−65	92	27	191	83	109	
3 Control period	May 21	12	590		6 4	36	95	131	116	65	51	16 per cent cream, 390 cc. Water, 730 cc Lactose, 50 gm.
	" 22	12	500		6 4	30	91	121	100	60	40	
	" 23	12	540		6 4	37	76	113	112	63	49	
	Averages	12	543	1 20	6 4	34	86	120	110	63	47	
Experimental period	May 25	12	590		5 9	52	100	152	116	61	55	Administered 100 cc of 0.1n HCl.
	" 26	12	600		5 9	60	119	179	116	63	53	No acid given
	Averages.	12	595	—	5 9	56	109	165	116	62	54	

Subject M, age 14 months, weight 6 5 kg

Period	Date											Notes
4 Control period	May 21	7	690		6 2	30	90	120	130	70	60	16 per cent cream, 230 cc. Water, 900 cc Lactose, 30 gm
	" 22	7	640		6 2	32	94	126	136	72	64	
	" 23	7	650		6 2	33	84	117	133	70	63	
	" 24	7	620		6 2	31	92	123	130	68	62	
	Averages	7	650	0 84	6 2	32	90	122	132	70	62	
Experimental period	May 27	7	680	—	8 2	−32	31	−1	131	71	60	4 gm of NaHCO₃ daily; begun May 26.

normal pH is managed by excretion of the phosphates in correct relative amounts.   Slight variations in acid production may be entirely compensated by variation in the relative amounts of the phosphates excreted, the ammonia factor remaining stationary. The acidity of the urine on normal diets may, for this reason, vary widely, the pH being frequently as great as when acidosis is present.   It is therefore impossible to obtain from the pH of the urine a dependable indication of the presence of an acidosis.

It is equally incorrect to assume that diets containing an unusually large amount of acid-producing elements, or which lead to the production of abnormal acids, will necessarily cause an acidosis   Acidosis cannot safely be considered indicated unless a depletion of the fixed alkali of the blood has been demonstrated. Denis and Minot[7] have been able to obtain with women subjects an excretion of creatine by giving diets unusually high in protein; which necessarily contain a large quantity of acid-forming elements.   They have found that the addition of sodium bicarbonate to this diet produces no appreciable effect on the creatinuria. This result shows very clearly that a wide variation in the acid-base value of a diet may bear no relation to creatine excretion.

We have thought it worth while to illustrate this point further by adding acid or base to the food of infants.   The results of these experiments are tabulated in Table I.   We present the results of a single experiment in which 100 cc of 0.1 N hydrochloric acid were given to an infant in its food, in divided amounts, during the course of 24 hours   There is a well marked effect on the factors concerned in acid excretion during the day the acid was given, and also on the day following.   The value for creatine excretion remains the same as during the control period.   We gave sodium bicarbonate freely in several instances, but did not find a change in the quantities of creatine eliminated.

We have been able to find a record of but one set of experiments in which creatine was measured in the presence of a demonstrated acidosis.   Sawyer, Stevens, and Baumann,[8] using as subjects male children, were able to produce quite regularly a depletion of the fixed alkali of the blood by giving a high fat and low carbo-

[7] Denis, W , and Minot, A S , *J Biol Chem* , 1919, xxxvii, 245

[8] Sawyer, M , Stevens, F A , and Baumann, L , *Am. J Dis Child* , 1918, xv, 1   ᴬ

hydrate diet. They found in all their experiments that during the period of acidosis there was a well marked increase in the excretion of creatine They were also usually able to obtain a lowering of the bicarbonate of the blood by giving a low calorie diet, with nearly always an increase in creatine excretion. They found, however, in three instances an increase in creatine excretion on the low calorie diet without a reduction of blood bicarbonate. Their results suggest a relationship of acidosis to creatinuria. Such an inference cannot, however, be drawn with certainty because of the wide variation in the diet of factors other than those related to acid production. The authors themselves express their belief that the creatinuria was not due to the acidosis *per se*. We would suggest that the increase in creatine excretion may have been due to an abnormal protein metabolism, caused, in one case, by an inadequate intake of protein and, in the other, by the absence of the protein-sparing effect of carbohydrate. As compared with its value on the normal control diet, the nitrogen in the urine was relatively high during the low calorie periods and absolutely high during the high fat intake periods

We would say in conclusion that variations in the acid-base value of normal diets cannot possibly bear relation to creatinuria in man for the reason that they are never of sufficient magnitude to disturb the normal acid-base equilibrium within the body. As regards the relation to creatine excretion of acidosis produced by abnormal diets, we do not find in the literature satisfactory evidence indicating acidosis *per se* as a factor concerned in causing or increasing creatinuria.

The Elimination of Ingested Creatine and Creatinine in Infants.

It is well established by the original investigations of Folin[9] and of Klercker[10] that large amounts of creatine may be ingested by adult men with a failure of creatinuria. Moreover, doses of creatine, which fail to produce creatinuria on a low protein diet, cause a creatine output on a high protein intake Only a small percentage of the ingested creatine appears in the urine

[9] Folin, O , Festschrift fur Olof Hammersten, Upsala, 1906
[10] af Klercker, K. O , *Beitr Chem Physiol u Path* , 1906, viii, 59; *Biochem Z* , 1907, iii, 45

in any case, and for the most part, the ingested nitrogen remains unaccounted for in the urine

Mellanby[11] suggested that the results given above may be due to the action of intestinal bacteria in destroying the ingested creatine.   In fact, Twort and Mellanby[12] have isolated a bacillus from the human feces which destroys creatine.   Hence, according to these authors, in creatine-feeding experiments this factor must be taken into account.   While the validity of this observation cannot be denied, it must be pointed out that according to more recent investigations, in which the creatine is introduced parenterally, the original contention of Folin and of Klercker is confirmed. Lyman and Trimby[13] find that 76 per cent of the creatine nitrogen, injected subcutaneously into man, remains unaccounted for in the urine.   Meyers and Fine,[14] and Rose and Dimmitt,[15] after ingestion, as well as Lyman and Trimby[13] after injection of creatine, believe that they found an increased output of urinary creatinine which they attribute to converted creatine.   In this finding they stand in opposition to the results of Folin[9] and of Klercker.[10]

When creatinine is fed to adult men, observers are agreed that greater amounts are eliminated than is the case with creatine Folin[9] and Rose and Dimmitt[15] report recovery of 80 per cent of the creatinine ingested.

Krause[16] investigated the fate of exogenous creatine in two girls (aged 6 and 11 years), and two boys (aged 5 and 8 years), the older of each sex was not excreting creatine.   He finds that, in children, relatively small amounts of creatine (0.3 to 0.35 gm) lead to an increased creatinuria if one already existed, or to a creatinuria where there was none previously.   The author concludes that in children the power of "assimilating" creatine, if measured in absolute amounts, is less developed than in adults   Further, this is true even when the power of "assimilating" creatine is calculated per kilo of body weight; and the younger the child the less ingested creatine can it retain

[11] Mellanby, E , J Physiol , 1907–08, xxxvi, 447

[12] Twort, F W , and Mellanby, E , J Physiol., 1912, xliv, 43

[13] Lyman, J F , and Trimby, J C , J Biol Chem , 1917, xxix, 1

[14] Meyers, V C , and Fine, M S , J. Biol Chem , 1915, xxi, 377

[15] Rose, W. C , and Dimmitt, F. W , J Biol Chem , 1916, xxvi, 345

[16] Krause, R A , Quart J Exp Physiol , 1914, vii, 87

The investigation to be presented herewith was instituted in order to test the effect of ingested creatine upon the creatinuria of infants, not only because of the interest attached to the data thus obtained, but also since they are necessary in connection with results presented in the paper which follows.

Both subjects used were of the male sex and creatine was a constant urinary constituent The creatine and creatinine administered were dissolved in the total amount of milk to be fed during the day.   Hence, the subject received but a small amount of creatine or creatinine at each feeding.

The creatine was a pure preparation free from creatinine and contained 1 mol of water of crystallization.   The amounts noted in the protocols, however, are calculated as anhydrous.   The creatinine was likewise a pure preparation isolated from urine.

EXPERIMENTAL.

In the experiments detailed in Table II, it is apparent that even on a low protein diet (Experiments 1 and 5), from 12 to 16 mg. per kilo, or an absolute amount of 88 mg., suffice to cause an increased urinary creatine.   No attempt is made to determine the least amount of ingested creatine which would lead to such an increase, but indications are that it would be very small.

As regards the completeness of excretion, it will be noted that in Experiments 1, 2, and 5, on a low protein diet, 24 to 34 per cent of the ingested creatine is recovered in the urine during the first 24 hours.   It must be recalled that, in these experiments, the creatine administered was mixed with the food and given throughout the day with each feeding.   As a result it is found that the urine of the 2nd day still contains extra creatine   Hence, if the total extra creatine excreted in 2 days is calculated, we obtain figures of from 35 to 58 per cent

In Experiment 3, the subject is on a high protein level   The afterperiod is considerably lengthened in order to study more completely the lag in creatine excretion.   In this experiment, taking the 1 day foreperiod as the normal output, in the first 24 hours there is an extra excretion of 52 per cent of the creatine ingested.   Moreover, it will be seen that the urinary creatine continues to be high for at least 3 days after the day of feeding.

TABLE II

Effect of Ingested Creatine and Creatinine upon Urinary Creatine and Creatinine.

Subject G, age 10 months

| Experiment No | Date. | Weight | No of days on diet | Protein per day | Total N | Urine | | | | Remarks |
						Total creatinine	Preformed crnt inine	Creatine as creatinine	Creatine	Diet
	1917	*kg*		*gm*	*gm*	*mg*	*mg*	*mg*	*mg*	
1 Control period	May 31	7.2	18	12	1.57	136	71	65	75	Low protein diet {16 per cent cream water}. Urine contained acetone during 3 days of experiment
Experimental period	June 1			12	—	156	71	85	90	Administered in food 0.088 gm of creatine. Extra creatine excreted on 1st day = 24 mg = 27 per cent of ingested creatine
	" 2			12	—	143	72	71	82	Total extra creatine excreted in 2 days = 31 mg = 35 per cent of ingested creatine
2 Control period	June 9	7.7	26	12	0.84	137	75	62	72	Low protein diet {16 per cent cream lactose water}
Experimental period	June 10			12	—	215	75	140	162	Administered in food 0.264 gm of creatine. Extra creatine excreted on 1st day = 90 mg = 34 per cent of ingested creatine
	" 11			12	—	176	76	100	116	Total extra creatine excreted in 2 days = 134 mg = 51 per cent of ingested creatine

		8 2	9	40	5 16	194	87	107	124	
3 Control period.	June 25	8 2	9	40	5 16	194	87	107	124	High protein { whole milk diet
Experimental period	June 26			40	—	317	93	224	260	Administered in food 0 264 gm of creatine Extra creatine excreted on 1st day = 136 mg = 52 per cent of ingested creatine
	" 27			40	—	242	86	156	181	Total extra creatine excreted in 2 days = 193 mg = 73 per cent of ingested creatine
	" 28			40	—	233	95	138	160	Total extra creatine excreted in 3 days = 229 mg = 87 per cent of ingested creatine
	" 29			40	—	222	89	133	154	Total extra creatine excreted in 4 days = 259 mg = 98 per cent of ingested creatine
	" 30			40	—	204	88	116	135	Total extra creatine excreted in 5 days = 270 mg = 102 per cent of ingested creatine
4 Control period	June 19 / " 20	8 2	2	40 / 40	5 30 / —	218 / 210	83 / 84	135 / 132	157 / 153	High protein { skim milk diet
Experimental period	June 21			40	—	314	185	129	150	Administered in food 0 2 gm of creatinine Extra creatinine excreted 1st day = 101 mg = 50 per cent of ingested creatinine
	" 22			40	—	234	107	127	147	Total extra creatinine excreted in 2 days = 124 mg = 62 per cent of ingested creatinine.

TABLE II—*Continued*

Experiment No	Date	Weight	No of days on diet	Protein per day	Total N	Total creatinine	Pre-formed creatinine	Creatinine creatinine	Creatine	Remarks Diet
		kg		*gm*	*gm*	*mg*	*mg*	*mg*	*mg*	
	1917									
							Subject S, age 8 months			
5 Control period	June 4	5·4	3	6	0·50	68	36	12	14	Low protein { 16 per cent cream, lactose, water
	" 5			6	—	68	36	12	11	
Experimental period	June 6			6	—	81	51	30	35	Administered in food 0·088 gm of creatine. Extra creatine excreted on 1st day = 21 mg = 24 per cent of ingested creatine.
	" 7			6	—	91	53	38	11	Extra creatine excreted in 2 days = 51 mg = 58 per cent of ingested creatine

These increases are greater than the experimental variations from day to day   In the first 2 days 73 per cent of the ingested creatine is excreted, in 3 days 87 per cent, and in 4 days 98 per cent.   The figure on the 5th day is still slightly higher than the foreperiod, so that the total excretion is 102 per cent.   Allowing 10 per cent, a very liberal allowance, for a possible variation in the normal excretion, the figure for creatine on the 5th day may be considered as a return to the normal.   Therefore, the average normal excretion is between 124 and 135 mg. (130 mg.).   On this basis we get a total extra excretion of creatine of 235 mg , or 89 per cent of the amount ingested.

Experiment 4 presents an isolated experiment on creatinine ingestion.   In a period of 2 days, 62 per cent of the ingested creatinine appears in the urine.   The daily output during this time is still not down to the normal figure of the foreperiod, hence it is possible that, had the period been lengthened, more creatinine might have been excreted

These experiments show no evidence of the conversion of ingested creatine into creatinine or *vice versa*.

<center>DISCUSSION.</center>

The results given above show: first, that in the infant small amounts of ingested creatine lead to an increased urinary output Second, there is evidence that in infants the ingested creatine is nearly or completely eliminated during a period of several days. Third, from a comparison with experiments in the literature of the behavior of creatine ingested by adult men with the behavior in the infants studied by us, the following points are suggested   (a) smaller absolute amounts of ingested creatine lead to urinary excretion of creatine in infants than is the case with adult males, (b) ingested creatine is more completely excreted by the infant than by the adult male; (c) although the comparison presents greater difficulties of demonstration, there is an indication that, per kilo of body weight, smaller quantities of ingested creatine lead to excretion of creatine in infants than in adult males

In Experiments 1 and 5 (Table II), where the lowest protein level obtains, it is found that from 12 to 16 mg of creatine per kilo of body weight or an absolute amount of 88 mg is sufficient

to cause an increased urinary creatine of from 35 to 58 per cent
in 2 days   Under similar conditions in the adult male, Folin[9]
found it necessary to administer 6 gm. (in three portions) before
excretion of creatine is obtained, and even then but 16 per cent
of the amount ingested is recovered.   Klercker[10] took 2.06 gm.
(22 mg per kilo) of creatine on a low protein diet with failure of
excretion.   Plimmer, Dick, and Lieb[17] are compelled to administer
2.5 gm. of creatine to an adult man, on an average protein intake,
before creatinuria occurs.   This amounts to 34.7 mg. per kilo of
body weight.

On a high protein level (Experiment 3), the excretion in our
subject is somewhat greater in an equal interval of time than on
a low level of protein intake.   Of the ingested creatine (264 mg
or 32 mg. per kilo of body weight), 73 per cent is eliminated in 2
days, and nearly, if not all, in an interval of 5 days.   On a high
protein level Folin[9] found that ingestion of 5 gm of creatine (in
three portions) leads to the excretion of 36 to 54 per cent of the
amount ingested.   Klercker finds that under like conditions when
2.59 gm. are taken (28 mg per kilo) but 30 per cent of the ingested
creatine is excreted

The results obtained by us are in accord with those obtained
by Krause[16] on older children, although this author did not observe
the complete excretion of creatine which is indicated in our
experiment.

In the normal adult (Folin,[9] Klercker,[10] and Meyers and Fine[14]),
there is no such lag leading to a complete excretion of creatine as
is observed in Experiment 3

Powis and Raper[18] found that upon feeding 206 mg of creatine
to a girl 4 years old, with amyotonia congenita, there was an
increased creatine excretion for 48 hours following its adminis-
tration, leading to a nearly total elimination of the ingested
creatine.

Determinations of creatine tolerance for children and adults of
both sexes have not been made, and from scattered observations
in the literature, accurate comparisons are impossible   However,
on the basis of our experiments, it seems justifiable to say that

[17] Plimmer, R H A, Dick, M, and Lieb, C C, *J Physiol*, 1909–10, xxxix, 98
[18] Powis, F, and Raper, H S, *Biochem J*, 1916, x, 363

in the infant the tolerance is much less than in the adult, and, furthermore, excretion is more complete in the infant than in the adult.

The significance and bearing of these results on recent work will be brought out in the next paper.

CONCLUSIONS

1. Variations in the acid-base intake have no effect on the creatinuria of infants.

2. There is no satisfactory evidence that acidosis *per se* is a factor in the production of creatinuria.

3. Small amounts of ingested creatine lead to an increase of the creatinuria in infants. In a single experiment, on a high protein diet, a practically complete elimination of ingested creatine was observed.

4. There is evidence that the infant differs radically from the adult in its behavior towards ingested creatine.

A STUDY OF CREATINURIA IN INFANTS.

II. RELATION OF PROTEIN INTAKE TO URINARY CREATINE.

By JAMES L. GAMBLE and SAMUEL GOLDSCHMIDT

(From the Laboratory of the Department of Pediatrics, and the Hunterian Laboratory of Experimental Pathology, Johns Hopkins University, Baltimore.)

Effect on Creatine Excretion of Different Levels of Protein Intake Obtained by Giving Milk or Milk Dilutions.

The relation to creatinuria of various dietary factors has been suggested and investigated   Recent investigations seem to indicate quite definitely a relation of creatinuria to the size of the protein intake.[1]   In as far as these observations apply to infants, investigators are confronted with the difficulty of administering a creatine-free diet.   We shall present data in this paper which show that, in the infant on a milk diet, the protein intake *per se* is not entirely responsible for the increased output of creatine obtained by increasing the intake of milk.

One of us[2] several years ago, during a study of the nitrogenous end-products found in the urine of an infant at different levels of protein intake, noted that during the period when the least amount of protein was given there was an almost complete disappearance of creatine from the urine   As the quantity of protein in the food was increased, the amount of creatine in the urine rose steadily.   The creatinine excretion preserved throughout the experiment a closely constant value.   During all the experimental periods the infant received a normal caloric intake, and the nitrogen balance was positive.   The size of the protein intake, as a factor concerned in determining the quantity of creatine in the urine,

[1] Denis, W , *J Biol Chem* , 1917, xxx, 47   Denis, W , and Kramer, J. G., *ibid* , 189   Denis, W , and Minot, A S , *ibid* , 1917, xxxi, 561.
[2] Talbot, F B , and Gamble, J L , *Am J Dis Child* , 1916, xii, 333.

seemed clearly indicated by this experiment.   Folin and Denis[3] had previously suggested that this effect might be obtained in children by varying the quantity of protein given.   They based their assumption on the theory that the normal creatinuria of children is due to a much higher level of protein consumption in proportion to body mass than is the case with adults.

We undertook some time ago to confirm these findings by repeating the experiment with several infants.   Soon after we had begun our work, Denis and Kramer[1] published the results of similarly devised experiments, using as subjects several children and one infant.   They found a wide difference in the amount of creatine in the urine when the excretion on high and low protein diets was compared   They conclude, "that the amount of creatine found in the urine of children is directly dependent on the intake of protein, being high when large quantities of protein (creatine-free) are ingested, decreasing and in some cases disappearing entirely when the child is fed a diet of an extremely low protein content " The protein constituents of the high protein diet given the children were eggs, cheese, gelatin, and milk.   A mixture of whole and fat-free milk constituted the high protein food of the infant.   During the low protein period, the infant received 40 per cent cream, oatmeal water, and lactose

In the experiments on infants, to be reported in this paper, different levels of protein were obtained in quite the same way, except that for the low protein periods we used a dilution of 16 per cent cream   We therefore did not feed so small a quantity of protein as did Denis and Kramer,[1] which possibly accounts for the fact that we did not obtain in any instance a complete disappearance of creatine from the urine.   A proper caloric value was obtained in the cream dilutions by the addition of lactose.   The methods employed have been described in the preceding paper.

Results of Experiments.

The results of our experiments are given in Table I   They are substantially in agreement with those of Denis and Kramer.[1]   A high excretion on whole milk and a low excretion on diluted cream is obtained in both instances.   Dilutions of whole milk give an

[3] Folin, O , and Denis, W , *J Biol Chem* , 1912, xi, 253

TABLE I

Effect on Creatine Excretion of Increasing the Quantity of Milk Given.

Food	Fore-period	Date	Urine			Weight
			Nitrogen	Creatinine	Creatine as creatinine	
Experiment 1 Subject M, age 14 months, underweight						
1	*days*	*1917*	*gm*	*mg*	*mg*	*kg*
16 per cent cream, 224 cc Water, 896 cc Lactose, 56 gm (½ whole milk)	5	Mar 22 " 23 " 24 " 25		70 72 70 68	60 64 63 62	6 5
7 gm protein			0 84	70	62	
2						
½ whole milk + lactose 47 gm , 1,120 cc	5	Apr 2 " 3 " 4 " 5		76 70 68 69	75 68 61 64	6 6
18 gm. protein			2 14	71	67	
3						
Whole milk + lactose 20 gm , 1,120 cc	2	Apr 18 " 19 " 20		72 71 76	97 93 89	6 7
36 gm protein			4 56	73	91	
4						
Whole milk + lactose, continued	14	Apr. 30 May 1 " 2 " 3 " 4		80 79 81 82 74	110 107 99 120 117	6 7
			4 62	79	111	
5						
Whole milk + lactose, continued	22	May 8 " 9		81 81	125 124	6 8
			5 08	81	124	

TABLE I—*Concluded.*

Effect on Creatine Excretion of Decreasing the Quantity of Milk Given.

Food	Fore-period	Date	Urine			Weight
			Nitro-gen	Creat-inine	Creatine as creat-inine	
Experiment 2 Subject G, age 10 months, normal infant						
1	*days*	*1917*	*gm*	*mg*	*mg*	*kg*
Whole milk + lactose 20	13	May 11		84	109	6 8
gm , 900 cc		" 12		82	108	
30 gm protein			4 37	83	108 .	
2						
16 per cent cream, 390 cc	2	May 15		72	79	6 5
Water, 730 cc		" 16		71	69	
(⅓ whole milk)		" 17		69	53	
		" 18		61	49	
12 gm protein.			1 86	68	63	
3						
Same + lactose, 50 gm	8	May 21		65	51	6 9
		" 22		60	40	
		" 23		63	49	
			1 20	63	47	

intermediate value. It should be noted, however, that in the case of Subject M there is still a considerable excretion of creatine when the food given is cream diluted four times. We are inclined to doubt the possibility that creatine could have been made to disappear by giving still less protein.

The fact that the increase in creatine excretion takes place quite gradually, on changing from a low to a high protein intake, appears in the tables. In the case of Subject M, the increase in creatine excretion on a high protein intake is followed over a period of 3 weeks, and even then we cannot be certain that it has reached a stationary value. It will also be noted that on changing from a high to a low protein intake (Subject G) the decrease in creatine excretion, while it takes place more rapidly, nevertheless requires

a period of days to reach a fixed level. In order to indicate the relation of this time factor to our results, we have placed in the tables, in the column marked "foreperiod," the number of days the infants were on a given protein intake before the observation period was begun.

Besides the increase in creatine excretion as the level of protein intake is raised, there is also a gradual but definite increase in creatinine elimination (Subject M). Greatly lowering the protein intake produces a well marked decrease in creatinine excretion (Subject G). A relation of protein intake to creatinine excretion is not discernible in the tables of the results of Denis and Kramer's[1] experiments.

Effect on Creatine Excretion of Varying the Amount of Whey Given.

Different levels of protein intake were obtained in the experiments just described by giving whole milk, whole milk diluted, or 16 per cent cream diluted. The infants, therefore, received various amounts of cow's milk. The resultant effect on creatine excretion is evident. However, from the results of experiments described below, it appears clearly that creatine excretion in infants, fed on cow's milk, cannot be related directly to protein intake *per se*.

Although milk has been regularly and largely used as a constituent of creatine-free diets, it has long been known that milk is not creatine-free The quantity of creatine in cow's milk has been considered so small as to bear no appreciable relation to creatine excretion. In the case of infants, however, whose sole food is milk, and who take a relatively large volume of it, it would seem that its creatine content ought to be considered.

Using the method of Wilson and Plass,[4] we have obtained figures for preformed creatinine of $\pm$ 1.0 mg., and for creatine of 6 to 11 mg. per 100 cc. of cow's milk. Folin's method[5] may give results equally high. The older infants, with whom we experimented, received during the period of high protein feeding more than a liter of milk. If the value of creatine content given above is approximately correct, variation in the quantity of preformed

[4] Wilson, D W , and Plass, E D , *J Biol Chem* , 1917, xxix, 413
[5] Folin, O , *J Biol Chem* , 1914, xvii, 475

cieatine in the food would be sufficient to account for the diffeience between the quantities of creatine in the urine, when large and small amounts of cow's milk weie given   We very much doubt their coriectness.

Denis and Minot[6] have recently reported that the values obtained by Folin's method are twice too high   We submit, however, that it is extremely difficult to determine the quantity of creatine present in milk   We have,· by various devices, tried to obtain a dependable coloi of sufficient intensity to read accurately, but have been quite unable to attain the conviction that we could measure the quantity of creatine present with even approximate accuracv   We have had no better success with the recently published method of Denis and Minot [6]

In an experiment described in the preceding paper, it was found that creatine fed to an infant was excreted appaiently quantitatively   This result greatly increased our suspicion that the preformed creatine present in cow's milk may be a large factor in the creatine excretion of infants   Having decided that we were unable to test this point directly by measuring the creatine content of milk, we undertook to obtain indirect evidence by observing the effect on creatine excretion of varying the amount of whey in the food   Creatine may reasonably be supposed to be in solution in milk   If this is true, its quantity in the infant's food will be proportional to the amount of whey present.

Results of Experiments

The results of these experiments aie given in Table II.   In Experiments 1 and 2, three experimental diets were used.   The infant was first given diluted cream, then undiluted whole milk, and, finally, diluted cream plus purified casein to such extent as to raise the protein value of the mixture to nearly that of undiluted milk.   In Experiment 1 the excretion of creatine falls to the same low value on the diluted cream plus casein diet (Period 3) as when diluted cream alone is given (Period 1), in spite of the fact that the protein intake on the former diet is three times that when diluted cream alone is given.   When the protein intake is increased by administration of skim milk (Period 2), the creatine excretion

TABLE II

Effect on Creatine Excretion of Varying the Quantity of Whey in the Food

Food	Fore-period	Date	Urine			Weight
			Nitrogen	Creatinine	Creatine as creatinine	
Experiment 1	**Subject G, age 10 months, normal infant.**					
1	days	1917	gm	mg	mg	kg
16 per cent cream, 390 cc	8	May 21		65	51	
Water, 730 cc		" 22		60	40	
Lactose, 50 gm		" 23		63	49	
12 gm protein			1 20	63	47	6 9
2						
Skim milk, 1,120 cc	6	June 19		83 .	135	
		" 20		84	132	
40 gm protein			5 30	84	133	8 2
3						
16 per cent cream, 280 cc	5	July 6		79	44	
Water, 840 cc						
Casein, 30 gm		" 7		83	48	
Lactose, 60 gm						
38 gm protein			3 84	81	46	8 2
Experiment 2	**Subject S, age 8 months, undernourished**					
1						
16 per cent cream, 168 cc	3	June 5		56	12	
Barley water, 672 cc						
Lactose, 60 gm		" 6		56	12	
(¼ whole milk.)						
5 gm protein			0 50	56	12	5 4
2						
Whole milk, 900 cc	10	June 18		56	59	
		" 19		55	58	
30 gm. protein			3 78	56	58	5 5
3						
16 per cent cream, 230 cc	8	July 5		55	48	
Barley water, 760 cc						
Casein, 20 gm		" 6		53	47	
Lactose, 45 gm						
27 gm protein			3 03	54	47	5 9

TABLE II—*Concluded*

Food	Fore-period	Date	Urine			Weight
			Nitro-gen	Creat-inine	Creatine as creat-inine	
Experiment 3 Subject X, age 16 months, underweight						
1	*days*	*1919*	*gm*	*mg*	*mg*	*kg*
Whole milk, 1,180 cc	5	Apr 2		83	80	
		" 3		81	78	
		" 4		81	81	
Nitrogen, 5 34 gm			4 41	82	81	5 9
2						
Water, 1,050 cc	7	Apr 22		81[1]	18	
Curd from 1,500 cc milk		" 23		90	22	
Dextri-maltose, 70 gm		" 24		91	19	
Nitrogen, 4 20 gm			2 60	88	20	5 9
Experiment 4 Subject R, age 2 yrs , underweight.						
1						
Whole milk, 1,120 cc	3	Mar 20		78	93	
		" 21		81	89	
Nitrogen, 5 61 gm			4 40	80	91	6 8
2						
16 per cent cream, 320 cc	2	Apr 9		93	105	
Whey, 800 cc						
Lactose, 24 gm		" 10		99	104	
Nitrogen, 2 26 gm			1 43	96	104	7 2
3						
16 per cent cream, 320 cc	5	Apr 25		91	34	
Water, 800 cc						
Casein, 24 gm		" 26		89	34	
Lactose, 24 gm						
Nitrogen, 4 35 gm			3 21	90	34	6 9
Experiment 5. Subject C, age 4 months; slightly underweight.						
1						
Breast milk, 620 cc	1	Apr 4		46	10	
		" 5		47	11	
		" 6		45	11	
Nitrogen, 1 01 gm			0 42	46	11	4 5
2						
16 per cent cream, 134 cc.	3	Apr 19		55	51	
Whey, 536 cc		" 20		60	40	
Lactose, 7 gm		" 21		69	41	
Nitrogen, 1 15 gm			0 81	61	44	4 6

[1] Computed from a 12 hr specimen

is markedly increased   In Experiment 2, with a similar experi-
mental procedure, the same result is not obtained; the creatine
excretion remains nearly as high on the diluted cream plus casein
diet (Period 3), as during the whole milk period (Period 2).   In
Experiment 4, however, there is also found a much lower creatine
excretion on a cream plus casein mixture than occurs when whole
milk is fed   Although the protein intakes are not exactly the
same, the difference is slight, and, furthermore, the differences in
creatine output are out of proportion to the slight variation in
protein. '

In another experiment (No. 3), four times as much creatine is
found in the urine on a whole milk diet as when curd without
whey is given.   The effect of diluting cream with whey is
tested in Experiment 4 and it is found that somewhat more
creatine is excreted than when the food is whole milk, containing
more than double the amount of protein

We obtained with a 4 months infant a breast milk period (Ex-
periment 5) and find the excretion of creatine low.   Breast milk
protein is, in contrast with cow's milk protein, largely composed
of whey proteins.   This infant was then given a cream and whey
mixture of nearly the same nitrogen value as breast milk, and
containing casein and whey in approximately the same proportions
as breast milk.   The creatine excretion on this food is four times
as great as on breast milk.   This observation suggests that cow's
milk may contain a great deal more creatine than breast milk
Inference from the results of this experiment is difficult, however,
from the fact that the urine during the cream and whey period
contains double the amount of nitrogen found when the food was
breast milk, although the nitrogen intake was the same

Creatinine excretion is increased in Experiment 1, when the food
was changed from diluted cream to skim milk, and the same effect
is obtained in Experiment 4, when whey was added to the
cream.   In both instances the higher value for creatinine excre-
tion is maintained when the diet was changed to a protein intake
composed mostly of casein, although on this diet there is, in
these experiments, a marked decrease in creatine excretion.   No
effect on creatinine was observed in Experiment 2.

Discussion of Results.

The most obvious inference to be obtained from these results is that creatine contained in cow's milk may be a large factor in the creatinuria of infants fed on modification of cow's milk. We are altogether uninformed as to the actual value for creatine in cow's milk, and therefore admit the possibility of the presence in the whey of a precursor of creatine   One point clearly brought out is that the creatinuria of infants cannot be directly referred to the total protein value of the milk given.   We do not wish to argue that the values for creatine excretion obtained by Denis and Kramer[1] in children may have been due to the milk portion of the high protein diet used.   We feel, however, that the as yet undetermined value for the creatine content of milk makes somewhat uncertain the interpretation of the significance of creatinuria when milk forms part of a supposedly creatine-free diet

The increased creatinine excretion on whole milk and on cream and whey may very well have been due to the larger amount of creatinine in the food than was the case when diluted cream was given.   The fact that the creatinine excretion remained high, when the high protein intake was composed nearly altogether of casein (Experiments 1, 2, and 4), requires some other explanation. We can only suggest with diffidence that it is conceivable that an increase in endogenous metabolism may have been caused by the effect on basal metabolism of the high protein intake.   The specific dynamic effect of protein has been shown to be very large in infants.[7]

Steenbock and Gross[8] have reported an increase of creatinuria in starving pigs when they are given casein   The results of our experiments on infants are not in agreement with this finding. Such a discrepancy of result is not, however, surprising when the wide difference in experimental conditions is taken into consideration.   The basis of Steenbock and Gross' experiments is a starving metabolism, whereas the subjects of our experiments received an adequate caloric intake.

    [7] Howland, J , *Tr 15th Internat Congr Hyg and Demog* , 1913, ii, pt 2, 438   Hoobler, B R , *Am J Dis Child* , 1915, x, 153
    [8] Steenbock, H , and Gross, E G , *J Biol Chem* , 1918, xxxvi, 265

SUMMARY.

'We piesent the results of experiments which indicate that the creatine excretion of infants bears a relation to the quantity of cow's milk fed, and also results of other experiments which indicate that the quantity of whey given is more directly related to the degree of creatinuria than is the total protein value of the food.

On the assumption that preformed creatine in milk modifications is in proportion to the quantity of whey present, our results suggest that the ingestion of creatine is probably a large factor in the creatinuria of infants fed on cow's milk. The desirability of a more definite knowledge of the creatine content of cow's milk is indicated.

Note —Since this paper was completed for publication Denis and coworkers have published creatine and creatinine determinations on human and cow's milk (*J Biol Chem*, 1919, xxxviii, 453, xxxix, 47), using the method of Denis and Minot [6] The amounts found are smaller than those obtained by earlier methods, yet, on the basis of the above work, are sufficient to pioduce changes in urinary creatine upon varying the quantities of whole milk fed to infants   The suggestion above, from an isolated experiment, that cow's milk may contain moie creatine than human milk, is not borne out by Denis' figures

PLACENTAL FEEDING AND PURINE METABOLISM.

By VICTOR JOHN HARDING and ELRID G. YOUNG.

(From the Biochemical Laboratory, McGill University, Montreal.)

In a previous paper Harding and Fort[1] made the suggestion that
if the placenta played an active part in the nutrition of the fetus
such a rôle might perhaps be found in its influence on the purine
metabolism. This was suggested by the fact that their placental
preparation proved to be unusually rich in arginine when com-
pared with other organs of the human body, and also by the fact
that Ackroyd and Hopkins[2] had adduced evidence to show that
arginine and histidine could be regarded as the precursors of syn-
thetic purines in the growing rat.

EXPERIMENTAL

In order to obtain experimental evidence either for or against
the supposition, it was decided to institute a series of feeding ex-
periments in which the influence of the placenta could be con-
trasted with that of other proteins which might possibly serve as
reserve sources of one or more amino-acids for the fetus. Pla-
cental feeding was compared with muscle feeding, and the effect
on the excretion of allantoin and uric acid noted in the growing
dog (not in the adult). The puppy was chosen as the experimental
animal, as it gave us the opportunity to use the meat powder and
placenta in moderate quantities, moreover, the quantity of food
could be readily controlled, and a sufficient amount of urine was
available for daily analysis. In addition, we wished to obtain
evidence on the condition of creatinuria which is present in puppies
and kittens according to Closson,[3] and our experiments were thus

[1] Harding, V. J., and Fort, C. A., *J. Biol. Chem.*, 1918, xxxv, 29.
[2] Ackroyd, H., and Hopkins, F. G., *Biochem. J.*, 1916, x, 551.
[3] Closson, O. E., *Am. J. Physiol.*, 1906, xvi, 252.

designed for a double puipose. Our experiments on creatinuria will be reported in a later communication. The kitten was found to be unsuited as an experimental animal. Confinement in a metabolism cage soon causes loss of appetite in the most robust animal Even with the puppy it was found advisable to institute a "fiee" day every 3 or 4 days to insure a continuance of health. On the "free" day the puppy received the experimental diet but was not confined in the metabolism cage. The puppies used were usually between 3 and 4 months of age. Before the experiment they were kept on an initial purine-free diet, mainly of bread and milk Their weight was recorded at regular intervals and showed that they were making satisfactory growth before and during the experimental periods.

A preliminary investigation, in which a number of puppies was allowed to eat a diet of bread, milk, and potatoes *ad libitum*, and on which they made satisfactory growth, showed us that about 100 calories per kilo of body weight could be considered as ample. Our experimental diets were consequently based on this figure. The following diets were used.

Diet	Weight	Content			Calories
		Protein.	Carbohydrate	Fat	
	gm	*gm*	*gm*	*gm*	
Initial purine-free		16 5	62 6	8 6	404 3
Milk	200				
Bread	100				
Meat		15 6	75 0	0 3	373 8
Potato	250				
Dextrin*	35				
Meat powder	15				
Placenta		15 6	75 0	0 4	373 9
Potato	250				
Dextrin*	35				
Placenta powder	15				

* Dextrin was sometimes replaced by corn-starch and occasionally in part by cane sugar

The day's rations were divided into two portions, supplied in the morning and evening, and were greedily eaten by the puppies. The potatoes were always very thoroughly boiled, and whenever corn-starch was

used it was made into a paste before being mixed with the remainder of the diet. These details, although seemingly insignificant, proved to be essential to the success of the experiments

Meat Preparation.

The meat powder was prepared from lean round steak   All visible fat and connective tissue were cut away, and the meat was passed through a mincing machine.   The minced meat was then placed in boiling water for a period of 10 to 15 minutes   By means of cheese-cloth the coagulated mass was strained from the aqueous solution and much liquid fat.   It was then reminced, and boiled again for a second and a third period   By this time practically all the fat and most of the extractives had been removed After the last filtration, the remainder was spread out on coarse filter paper and rapidly air-dried at ordinary temperature   The brittle, dark brown material remaining was then ground to a fine powder in a mill and kept ready for use in tightly stoppered bottles   When wanted for use, the amount required was weighed out and allowed to absorb water before adding it to the potato and dextrin or corn-starch   A determination of nitrogen by the Kjeldahl method showed 13 6 per cent of the dried substance

Placenta Preparation

The placenta,[4] immediately on delivery, was placed in a jar of sterile salt solution, covered by a layer of toluene, and the whole brought to the laboratory within a few hours   The fresh placenta was first washed free from large masses of clotted blood   Then the umbilical cord and free membranes extending beyond the mass of chorionic villi were cut off as short as possible   The placenta was next placed ventral side up in a large basin and the remaining membrane dissected away   It was found possible by this means to remove practically all the large blood vessels   The remaining mass was thoroughly washed under the tap, then torn up into small pieces and again washed, and finally strained through cheese-cloth The product was then finely minced in a mincing machine, and the remaining blood was removed by placing this material in tall glass jars through which a stream of cold water constantly circulated   To prevent loss of material a piece of cheese-cloth was tied over the top of the jar   The washing occupied from 6 to 12 hours, depending on the condition of the placenta   The washed mass was filtered through cheese-cloth and then coagulated by immersion in boiling water, faintly acidified by acetic acid, for a period of 10 minutes   At this stage the coagulated placenta was again passed through a mincing machine and subjected to further boiling in water for another two periods of 10 minutes each   After the final boiling

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[4] The placentas were obtained from the Montreal Maternity Hospital, and our thanks are due to the authorities for their kindness and cooperation

the mass was filtered at the pump, washed with distilled water, and air-dried   It was then ground to a fine powder in a mill, so that it passed through an 80 mesh sieve, and was preserved in stoppered bottles until required.   About 1 kilo of material was thus prepared, representing forty-three placentas   A determination of nitrogen showed 13 7 per cent of the dry weight

Total Purine Determination

The two experimental diets are not purine-free   Both the meat and placenta contain nucleoproteins and water-soluble purines The total purine content of muscle is given by Burian and Schur[5] as about 0 055 gm of purine nitrogen per 100 gm of fresh tissue. Placenta has been examined by Wells and Corper[6] who found 0 057 gm of purine nitrogen in 100 gm. of fresh tissue.   Thus, in the fresh state, the two tissues have practically identical purine contents.   In both our diets, however, washing of the protein had been resorted to, and we felt it incumbent upon us to determine directly the purine content of our meat and placental powders.

30 gm of meat or placental powder were boiled under a reflux condenser with 1 liter of 6 per cent sulfuric acid for 18 hours   The residue was filtered off and submitted to a further hydrolysis with 500 cc of 5 per cent sulfuric acid for 12 hours   A slight residue from this hydrolysis was neglected   The filtrates were then neutralized separately by sodium hydrate and brought back to a condition of slight acidity with dilute acetic acid They were next separately treated at the boiling point with copper sulfate as a 10' per cent solution and saturated sodium bisulfite solution, added alternately until a pronounced precipitate of cuprous oxide was visible The filtrate from the second hydrolysis gave very little precipitate   The cuprous purine precipitates were united and decomposed in warm water by saturation with hydrogen sulfide   The filtrate and washings from this decomposition were next evaporated down to about 500 cc , and the purines again precipitated by copper sulfate and saturated sodium bisulfite solutions   The decomposition of the cuprous compounds was carried out as before, and the purine filtrates were concentrated to about 75 cc   This was washed into a 100 cc volumetric flask quantitatively and diluted to the mark   A determination of nitrogen in the solution was made on aliquot parts by the Kjeldahl method and the results calculated as purine nitrogen

30 gm of meat powder gave 0 0360 gm of N = 0 9 per cent of total N of meat

30 gm of placental powder gave 0 060 gm of N = 1 47 per cent of total N of placenta

[5] Burian, R , and Schur, H , *Arch ges Physiol* , 1900, lxxx, 241

[6] Wells, H G , and Corper, H J , *J Biol Chem* , 1909, vi, 469

The two experimental diets thus differed in their purine content by about 12 mg per day   This small difference is insufficient to influence our results, for the differences which we have observed are far beyond that figure   In order to follow the influence of the placenta feeding upon the purine metabolism, we have determined the excretion of allantoin and of uric acid.

Urine Analysis.

The total nitrogen was determined by the Kjeldahl-Gunning process.   The allantoin was determined by the process proposed by Plimmer and Skelton [7]   We have found this method, or rather our variation of it, very useful; and if the allantoin is not too small in amount, it gives an accurate determination   The Folin magnesium chloride method for the determination of urea hydrolyzes urea and allantoin into ammonia which, together with the preformed ammonia, is distilled into an excess of standard acid from an alkaline solution   The urea and preformed ammonia were determined by the urease method devised by Van Slyke and Cullen.[8] The difference gives the allantoin in terms of ammonia.   We have modified the Folin magnesium chloride method[9] to suit our own convenience   In its original form, in our hands, it always required constant attention   Our modification, though a little more time-consuming, can be left unattended while other work is proceeding.

Pipette 5 cc of urine into a 200 cc. Erlenmeyer flask, and add 5 cc of concentrated HCl from burette and 20 gm. of crystalline magnesium chloride, together with a small cube of paraffin wax about the size of a marble   The magnesium chloride should be ammonia-free or have its ammonia content determined by blank tests.   It has been our experience that the commercial salt is more likely to be ammonia-free than the so called c p high priced article   A reflux condenser about 45 cm in length and 1 cm in diameter is attached to the flask and a large safety thistle tube to the upper end of the condenser   The safety tube also carries at the top a stopper with a glass tube bent at right angles. Without these safeguards we found it impossible to avoid loss by

[7] Plimmer, R H A , and Skelton, R F , *Biochem J* , 1914, viii, 70, 641.

[8] Van Slyke, D D , and Cullen, G E , *J Biol Chem* , 1914, xix, 211.

[9] Folin, O , *Am J Physiol* , 1905, xiii, 46

spraying. Having the condenser jacket empty, a medium sized Bunsen flame is played over the bottom of the flask until the contents boil fairly vigorously. Regulating the flame to maintain this rate of ebullition, the evaporation is continued until marked frothing occurs. This takes about 5 to 10 minutes. By gradually reducing the size of the flame, the frothing is easily controlled, and the concentration is continued until the flame of 1 inch in height is reached. This heat just serves to keep the solution boiling and is maintained for a period of 2 hours. Occasionally very little frothing occurs, and in these cases the concentration is continued until each drop of liquid falling back from the condenser causes a marked. spattering of the contents of the flask. Boiling gently for 2 hours continues from this point. The concentration to be aimed at is just short of saturation of magnesium chloride at that temperature. We have found that the primary boiling hydrolyzes the urea, and the second the allantoin. During the second period of boiling under constant conditions, a stream of cold water is run through the condenser. During this second period the determination requires no attention, as there is no danger under these conditions of the liquid in the flask becoming alkaline. At the end of 2 hours, the flask is allowed to cool, and the ammonia determined as in an ordinary Kjeldahl operation, using 10 cc. of 10 per cent sodium hydrate to render the contents of the distillation flask alkaline.

In order to prove the accuracy of this procedure for the determination of allantoin, it was deemed advisable to make tests on various concentrations of pure urea and allantoin solutions. The urea was determined by the urease method of Van Slyke and Cullen, while the Kjeldahl method of total nitrogen estimation served to check the mixed solutions. 2 5013 gm. of pure urea were dissolved in 100 cc of distilled water and 0 5005 gm. of allantoin was dissolved in a like volume of water

Urea determinations. ·

1. By urease method on 0 5 cc of solution

HCl N/20 cc	NaOH N/20 cc	Urea N mg per cc.
10	1 65	
10	1 65	
10	1 58	
10	1 58	
Average.		11 69

2. By Kjeldahl method on 5 cc of solution

HCl N/10 cc	NaOH N/10 cc	Urea N mg per cc
50	8 73	
50	8 70	
Average	... .	11 69
Calculated		11 68

Urea plus allantoin

1 By modified method, using 5 cc of urea + 5 cc. of allantoin solutions.

HCl N/10 cc	NaOH N/10 cc	N mg per cc
55	6 95	
55	6 90	
Average		13 52

2. By Kjeldahl method

HCl N/10 cc	NaOH N/10 cc	N mg per cc.
50	2 00	
50	2 10	
Average .		13 54
Calculated . . .		13 47
Allantoin determined . .		1 83
" calculated . .		1 79

The pure solutions were next diluted ten times with distilled water and the determinations repeated omitting the Kjeldahl estimations.

Urea determinations. .

1. By urease method on 0 5 cc. of solution.

HCl 0 01973 N cc	NaOH 0 02475 N cc	N mg per cc.
25	18 22	
25	18 24	
Average		1 157
Calculated		1 168

Urea plus allantoin

1. By modified method on 5 cc. of urea + 5 cc. of allantoin

HCl N/10 cc	NaOH N/10 cc	N mg per cc.
10	3 80	
10	3 85	
Average		1 342
Calculated		1.347
Allantoin determined		0 185
" calculated		0 179

TABLE I.

Urine Analysis

	Meat diet					Placenta diet				
Date	Total N	Urea N	Creatinine N	Allantoin N	Uric acid N	Date	Total N	Urea N	Creatinine N	Allantoin N	Uric acid N
1918	mg	mg	mg	mg	mg	1918	mg	mg	mg	mg	mg
Apr 6	1,099	784 3	25 38	24 98	1 45	Apr 13	Free day				
" 7	1,032	651 2	24 83	72 80	0 33	" 14	1,615	1,154	25 65	92 45	9 25
" 8	1,689	1,113	24 30		1 00	" 15	1,503	955 4	25 70	215 01	8 44
" 9	Free day					" 16	1,908	981 8	26 55	218 5	11 24
" 10	1,176	851 1	26 3	21 45	1 80	" 17	Free day				
" 11	1,153	825 4	22 36	96 00	0 0	" 18	1,764	1,214	32 08	165 5	9 77
" 12	1,274	890 4	27 87	60 24	2 70	" 19	1,433	1,030	32 15	43 05	6 42
Average				55 09	1 21					146 91	9 02

TABLE II.

Urine Analysis

	Meat Diet					Placenta diet				
Date	Total N	Urea N	Creatinine N	Allantoin N	Uric acid N.	Date	Total N	Urea N	Creatinine N	Allantoin N	Uric acid N.
1918	mg	mg	mg	mg	mg	1918	mg	mg	mg	mg	mg
Apr 6	1,033	626 1	20 27	42 55	0 23	Apr 13	Free day				
" 7	881	591 7	17 18	75 63	0 0	" 14	1,241	861 4	20 42	103 95	1 18
" 8	1,236	797 3	22 61	96 90	0 74	" 15	1,248	838 5	27 87	248 6	3 46
" 9	Free day.					" 16	1,319	864 4	24 05	107 2	4 18
" 10	1,172	828 7	24 38		0 0	" 17	Free day				
" 11	1,180	960 5	24 05	136 50	1 67	" 18	1,122	893 0	25 37	71 29	5 11
" 12	1,293	916 4	24 50	52 65	1 83	" 19	1,193	837 0	22 73	62.40	3 76
Average				80 85	0 74					118 70	3 54

TABLE III

Urine Analysis

Meat diet

Date	Total N	Urea N	Creat-inine N	Allan-toin N	Uric acid N
1918	*mg*	*mg*	*mg*	*mg*	*mg*
June 24	1,526	1,175	21 16	94 0	4 73
" 25	1,512	1,075	20 16	117 6	5 25
" 26	1,606	1,161	20 66	67 50	4 97
" 27	Free day				
" 28	1,602	1,050	20 34	141 7	5 14
" 29	Puppy not taking food				
Average	...	...	..	105 2	5 02

Puppy N

Placenta diet

Date	Total N	Urea N	Creat-inine N	Allan-toin N	Uric acid N
1918	*mg*	*mg*	*mg*	*mg*	*mg*
June 30	Puppy taking food normally				
July 1	1,637	1,056	21 39	267 1	9 48
" 2	1,758	1,224	24 23	228 0	8 27
" 3	1,541	988	23 10	241 0	8 46
" 4	Free day				
" 5	1,474	1,037	27 36	182 0	8 75
" 6	1,496	960	28 78	182 0	8 15
.....	...	...	.....	220 2	8 62

Meat diet

Date	Total N	Urea N	Creat-inine N	Allan-toin N	Uric acid N
1918	*mg*	*mg*	*mg*	*mg*	*mg*
July 7	Free day.				
" 8	1,263	858	24 83	123 3	5 75
" 9	1,097	768	26 20	112 0	5 11
" 10	1,343	974	29 64	98 7	5 71
.....	.			111 0	5 5

Uric acid was determined colorimetrically by the Folin-Macallum[10] method as modified by Benedict and Hitchcock.[11]   In the earlier part of the experiments, we were much troubled by turbidity arising in the solutions, but on using the modified Benedict uric acid reagent,[12] this difficulty disappeared.

As a check on the collection of urine, creatinine was determined by the micro method of Folin,[13] using creatinine zinc chloride as a standard, and picric acid purified according to the method of Folin and Doisy.[14]   The creatine determinations also formed an essential part of the investigation on creatinuria.   The urines were preserved under toluene and all analyses were carried out within 36 hours.   With the diets adopted we were untroubled by any fecal contamination of the urine.   Glucose was absent from all urines as judged by Benedict's qualitative test.   Likewise acetoacetic acid was never observed, although tested for by the nitroprusside method of Harding and Ruttan.[15]   The results are given in Tables I to III.

The protocols of the allantoin determinations and the weight records are appended in Tables IV, V, and VI.

TABLE IV.

Weight Records.

Date	Puppy W.	Puppy G	Date	Puppy N
1918	*gm*	*gm*	*1918*	*gm*
Apr 3	2,935	2,650	June 20	2,840
" 6	3,010	2,695	" 26	3,105
" 10	3,100	2,730	" 30	3,140
" 13	3,165	2,790	July 4	3,160
" 17	3,320	2,855	" 8	3,250
" 20	3,400	2,975	" 11	3,460
			" 15	3,270
			" 17	3,410

[10] Folin, O , and Macallum, A B., *J Biol. Chem* , 1912, xi, 265

[11] Benedict, S R , and Hitchcock, E H , *J Biol Chem* , 1915, xx, 619.

[12] Neuwirth, I , *J. Biol Chem* , 1917, xxix, 478 (Note 4)

[13] Folin, O , *J Biol. Chem* , 1914, xvii, 469

[14] Folin, O , and Doisy, E A , *J. Biol Chem* , 1916-17, xxviii, 349.

[15] Harding, V J , and Ruttan, R. F , *Biochem J* , 1912, vi, 445.

TABLE V

Allantoin Determinations

Date	Puppy W			Puppy G		
	HCl N/10	NaOH N/10	Allantoin N	HCl N/10	NaOH N/10	Allantoin N
1918	*cc*	*cc*	*mg per cc*	*cc*	*cc*	*mg per cc*
Apr 6	25	1 90	0 18	25	1 70	0 38
	25	1 87		25	1 68	
" 7	25	3 52	0 56	30	4 20	0 79
	25	3 50		30	4 20	
" 8				25	3 25	0 58
				25	3 20	
" 10	25	7 06	0 11			
	26	8 13				
" 11	25	13 80	0 30	25	13 80	0 42
	25	13 85		25	13 75	
" 12	25	9 50	0 25	25	9 20	0 27
	25	9 55		25	9 30	
" 14	50	4 58	0 86	25 5	2 85	0 63
	50	4 60		25 5	2 83	
" 15	75	18 75	2 47	25	3 43	1 28
	75	18 70		25	3 47	
" 16	25	28 30	0 87	25	8 50	0 46
	25	28 32		25	8 30	
" 18	25	0 37	0 77	25	6 20	0 36
	25	0 37		25	6 30	
" 19	25	3 69	0 2i	25	2 85	0 40
	25	3 65		25	2 90	

TABLE VI.

Allantoin Determinations.

Date	Puppy N			
	HCl N/10	NaOH N/10		Allantoin N
		1	2	
1918	*cc*	*cc.*	*cc.*	*mg per cc*
June 24	25	12 15	12 20	0 201
" 25	25	3 15	3 15	0 492
" 26	25	4 30	4 20	0 250
" 28	25	3 50	3 45	0 545
July 1	25	13 45	13 35	0 490
" 2	25	10 70	10 70	0 480
" 3	25	8 70	8 65	0 700
" 5	25	11 85	11 95	0 449
" 6	25	12 25	12 25	0 462
" 8	25	15 05	15 00	0 274
" 9	25	16 45	16 65	0 228
" 10	25	11 70	11 70	0 286

It is evident from an inspection of the figures given in Tables I to III that the feeding of placenta resulted in an increase in the excretion of both allantoin and uric acid. The smallest rise is shown by Puppy G, but even here the result is beyond the range of experimental error. Large fluctuations occur in the allantoin excretion; so large that in two cases the variations overlap in the two experimental periods, and at first led us to believe that there was something fundamentally wrong with our analytical technique. A revision of this, however, failed to show any serious error from this source except that it was realized afresh that extreme care must be taken over the allantoin determinations when there was only a small amount present As a further check, however, the uric acid was determined This, in the case of Puppies W and G, was made some weeks after the other analyses, but in Puppy N it was carried out concurrently with them As the uric acid figures show a similar increase during the period of placental feeding, we were confirmed in our opinion that the latter diet resulted in an increase in purine metabolism when contrasted with meat or muscle feeding. Furthermore, in Puppy N a return to the original meat diet led to a prompt drop in the allantoin and uric acid to their original level. The increase on the placenta diet is too large to be ascribed to the difference in purine content of the two diets In the meat diet 18 mg of purine N were supplied per day, in the placenta diet this was increased to 30 mg. per day, giving the small difference of 12 mg of purine N in favor of the placenta diet. This was the same for each puppy. The total increase in purine N estimated in the urine (allantoin N plus uric acid N) was 41 65, 99 63, and 118 60 mg respectively for Puppies G, W, and N

The possibility, too, of increased purine catabolism due to mere stimulation may also be dismissed Both diets possessed the same nitrogen content, both were equally well absorbed, and similar amounts of nitrogen were found in the urines of the two periods Both meat and placenta had been deprived of their extractives by water. It would appear then that the cause of the difference in the purine excretion under the two diets must be sought in their amino-acid make-up

In an important paper entitled "Feeding experiments with deficiencies in the amino-acid supply arginine and histidine as possible precursors of purines," Ackroyd and Hopkins,[2] as mentioned before, came to the conclusion that these two amino-acids either together or separately can act as the raw material for the synthesis of purines in the animal body.   It is a generally accepted fact that such a synthesis occurs, and naturally the presence of the glyoxaline ring in both purines and histidine, and a similarity of arrangement of carbon and nitrogen atoms in arginine and the pyrimidine nucleus, had long ago pointed to these two particular amino-acids as the probable source of such a synthesis   Abderhalden together with Einbeck[16] and Schmid[17] had attempted to obtain experimental evidence in favor of such a connection without success   Ackroyd and Hopkins owe their positive evidence to a better selection of experimental conditions.   They point out that in the adult animal such a synthetic process is of necessity at a minimum, and the evidence may well escape observation   Also that to supply an animal either in full nutrition or in a fasting condition with a large amount of an amino-acid suddenly is to supply it in excess of its current needs and to insure its catabolism by the most rapid path   If there now exists any alternative, a path involving a synthesis would be avoided [18]

In their evidence, Ackroyd and Hopkins observe the effect on growth and upon the excretion of allantoin in the growing rat of the withdrawal of arginine and histidine from the diet   The original diet, which was entirely adequate, contained caseinogen as its protein   The caseinogen was hydrolyzed by acid and tryptophane and cystine was added, thus rendering it complete in regard to its amino-acid make-up   On this diet the rats made satisfactory though not entirely normal growth   The removal of arginine and histidine led to a prompt loss in body weight and a

[16] Abderhalden, E , and Einbeck, H , Z physiol Chem , 1909, lxii, 322
[17] Abderhalden, E , Einbeck, H , and Schmid, J , Z physiol Chem , 1910, lxviii, 395
[18] The acceptance of this argument involves the assumption of the rapid adaptability of the organism   Otherwise the presentation of a large amount of a particular amino-acid would involve its metabolism by all paths in accordance with the active masses of material and the relative rates of the chemical reactions

fall in the allantoin excretion of 40 to 50 per cent. The withdrawal of only one of the two amino-acids, however, was followed by maintenance of weight, occasionally with a slight rise, and a drop in the allantoin excretion of about 17 per cent. It would seem as though arginine or histidine acted independently as an essential amino-acid, and functioned as a raw material for the synthesis of the purine ring in the growing rat. The fall in allantoin excretion is not a direct outcome of the drop in body weight occurring on the withdrawal of arginine and histidine, as the removal of trytophane from the diet occasions a loss in weight without a corresponding decrease in the allantoin excretion, although irregularities in the purine metabolism were noticed in one case

In attempting to contrast our two experimental diets, we were surprised to find no analysis of lean round steak made according to the Van Slyke method. We therefore utilized as the basis of our calculations the results of Thrun and Trowbridge[19] on a veal composite. In Table VII is given the amino-acid content of meat and placenta diets calculated in this way. We have also included the average results of two analyses of human skeletal muscle made by Drummond[20] The figures for the veal composite and human muscle do not differ markedly.

An inspection of the table shows that the meat diet is high in lysine as compared with the placenta diet and low in arginine. Taking its arginine and histidine content together, the placenta diet represents an increase of 50 per cent As no special function has as yet been discovered for lysine, and as the results show that an interpretation of our experimental figures must be sought in an increment in the placental diet, we are inclined to look upon our results as strongly supporting those of Ackroyd and Hopkins. We should also like to emphasize that in our experiments the uric acid excretion was increased, as well as the allantoin excretion, during the period of placental feeding. This we think important in view of the results of Benedict[21] on the purine metabolism of the Dalmatian hound in which the allantoin and the uric

[19] Thrun, W. E , and Trowbridge, P. F , J. Biol. Chem., 1917, xxxiv, 343

[20] Drummond, J C , Biochem J., 1916, x, 473

[21] Benedict, S. R , J. Lab. Clin. Med., 1916–17, ii, 1.

acid do not always follow parallel excretory paths   The increase in the uric acid shows unmistakably, in our minds, a synthesis of an actual *purine* ring.

Moreover, it should be again pointed out that in our experiments the animals were in full nutrition, as evidenced by their growth records.   The high arginine and histidine diet resulted in an increase in the synthetic processes   In this respect our experiments differed markedly from those of Ackroyd and Hopkins, and our results are not in accordance with some of the postulates expressed in that paper.

TABLE VII

Nitrogen partition	Placenta (Harding and Fort)[1]	Placenta diet	Veal composite* (Thrun and Trowbridge)	Meat diet	Human skeletal muscle (Drummond)
	per cent	*mg per day*	*per cent*	*mg per day*	*per cent*
Arginine N	22 54	450 8	13 3	266 0	11 17
Histidine N	3 12	62 4	5 0	100 0	4 25
Lysine N	7 22	144 4	13 5	270 0	13 15
Cystine N	1 36	27 2	1 00	20 0	0 85
Monoamino N	51 15		60 50		58 00
Non-amino N	5 85				4 30

* Calculated from figures given in Table II, by Thrun, W E , and Trowbridge, P F , *J Biol Chem* , 1917, xxxiv, 351

Lastly. how far these results may be taken as indicative of a function of the placenta, is a matter for further experimental inquiry.   It is certain that the high arginine content of the placenta, its ready hydrolysis by all classes of proteoclastic enzymes,[22] and the formation of allantoin and uric acid in feeding experiments with the young dog would go to show that one of its possible functions may be to act as a reserve store of arginine for purine synthesis in the fetus   The results of Lewis and Doisy,[23] who by feeding men diets high in arginine and histidine were unable to obtain any increase in the uric acid output, need not be considered as antagonistic to our view.   With adolescence may come a cessation or an impairment of the synthetic processes.

[22] Harding, V J , and Young, E G , *J Biol Chem* , 1918, xxxvi, 575
[23] Lewis, H B , and Doisy, E A , *J Biol Chem* , 1918, xxxvi, 1

SUMMARY.

From comparative feeding experiments on young dogs with equivalent diets containing muscle protein and placenta protein, the excretion of allantoin was found to rise markedly on the placenta diet.

The excretion of uric acid parallels that of allantoin and the conclusion is drawn that the comparatively large amount of arginine present in the placental diet is responsible for the increase in purine metabolism

A modification of the Plimmer and Skelton method of determining allantoin is described and proofs of its accuracy are given.

THE EFFECTS OF MALT AND MALT EXTRACTS ON SCURVY AND THE ALKALINE RESERVE OF THE BLOOD.

By J. F McCLENDON, W C C COLE, O ENGSTRAND, and J. E. MIDDLEKAUFF

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It has been well established that scurvy may be prevented or cured by eating fresh raw food.   The object of the present paper is to determine whether, in the entire absence of fresh foods, scurvy may be prevented or cured by malt products suitable for food for infants and adults.   Cereal grains may be easily transported and stored so as to preserve their sprouting capacity and there would be no necessity of a shortage of them, and hence no necessity of scurvy if the scurvy-ridden communities knew how to use them. Wiltshire has shown that human scurvy may be cured with sprouted beans, and Greig advocates their use in the army, but one of us has tested the sprouting of beans in army camps and under various climatic conditions and found them far less resistant to mould than are any of the cereal grains except maize.   Germination tests on many samples of beans bought from civilians showed that they were incapable of sprouting under any conditions   Army beans that showed over 90 per cent germination were attacked by mould if the temperature rose above 15° at night.

The relative importance of this work depends on the prevalence of scurvy, but we have not been able to obtain complete information on this subject.   During the Civil War 30,741 cases of scurvy among white troops were recorded   Bruntz and Spillmann designate "trench-foot" as a prescorbutic condition.   Many cases of scurvy have been diagnosed as such only after treatment on the supposition that the lesions were due to another cause

Durand finds that canned milk (which has been repeatedly shown to be deficient in antiscorbutics) causes dental caries in

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infants.   In examinations of young children whose infant histories
were known, 53 to 74 per cent showed dental caries following a
diet of canned milk, whereas breast-fed children showed 28 to 42
per cent dental caries   If the soundness of teeth depends on
antiscorbutics in the diet, the subject is one of greater importance
than it has generally been considered.

Soon after Holst and Frohlich in Norway began their work on
scurvy in guinea pigs, First, working in their laboratory, showed
that fresh sprouted barley is sufficient in antiscorbutic principles,
but that these properties are lost in the drying of the malt, as is
usually done in the beer industry   Weill and Mouriquand have
disputed the fact that sprouting barley contains antiscorbutic
substances but they admit that the young barley plant is effective
in this way.   Chick and Hume, and Cohen and Mendel have also
found antiscorbutics in sprouting grains (oats, barley).

Since barley grains yield an acid ash and Wright has claimed
that scurvy is the result of acidosis, we eliminated this possible
objection by determinations of the alkaline reserve of the blood
in scurvy as compared with that in health.   We used guinea pigs
because they are the classical animals for the study of scurvy and
also rabbits since they are particularly susceptible to acidosis.   In
order to control the ash of the food we took two equal weights of
barley, sprouted one part, and fed it to one animal, and fed the
other part, unsprouted, to an animal of the same size   Brown
has shown that the barley grain loses or gains no salts by steeping
as long as it is alive and that the salts gained by the steep water
come from the husk   Since guinea pigs do not eat the husk, we
assume that the one eating dry barley gets the same salts as the
one eating the same weight of sprouted barley.   The guinea pigs
were kept in cages of $\frac{1}{4}$ inch wire screen, with partitions so that
each guinea pig had a separate compartment   They learned to
drink out of inverted tubes filled with water.

The barley was sprouted by the drum method, large, wide-
mouthed glass jars serving as drums   The jars were placed on
two parallel steel shafts, 2 cm. in diameter, 200 cm. long, and placed
10 cm. apart   Both shafts were rotated in the same direction by
means of a small electric motor and worm-gear.   The friction of
the shafts on the jars caused them to rotate very slowly   Cool,
moist air was supplied by means of an aspirator pump and trans-

mitted by tubes projecting into the mouths of the jars. Two adjacent jars were placed with their mouths facing one another and air was supplied to both of them by means of a T-tube. Seed barley[1] was placed in a jar together with the amount of water it would absorb in 24 hours rotation, and at the end of that time it was washed, drained, and the rotation continued until the sprouting was complete. If the temperature within the jar approached 30° during the first 48 hours, death of some grains might occur, but after the first 48 hours (or, more correctly, after the
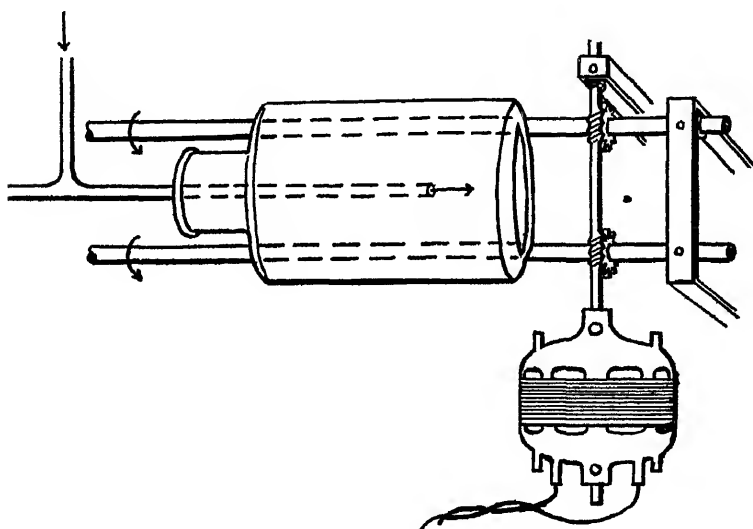
Fig 1.

acrospire appeared), the temperature might be raised to 30° with impunity. Since rise in temperature decreases the time required for sprouting, we never let it fall below 20°. The original design of the apparatus is shown in Fig. 1.

In determining the alkaline reserve, twelve guinea pigs and six rabbits were used. The animals were weighed every day, but the weight curves in this paper are smoothed so as to obliterate the confusing details since we were unable to compare the curves

[1] We are indebted to the cooperation of Professor A C Arny for the seed barley.

in their original form.   The weight curves of the guinea pigs are
shown in Fig. 2.   The spaces on the ordinate represent differences
of 100 gm. in weight, and the spaces on the abscissa intervals of
30 days.   The curves of the animals fed on sprouted barley (with
acrospire 1 inch long) are represented by unbroken lines, and the
curves of the animals on dry barley by broken lines   The animals
are numbered 1 to 6 beginning with the smallest (200 gm.) at the
left.   It may be seen that the animals on sprouted grain lost little
if anything in weight, whereas those on dry grain lost rapidly.
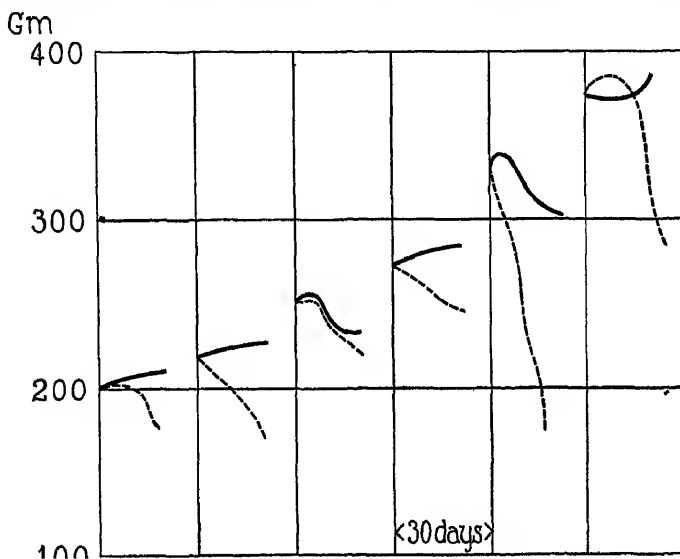
Fig. 2.

This was not due to lack of water as both sets were supplied with
water *ad libitum*, and the dry grain was fed in a dish of water.
The fifth guinea pig on dry grain died in 17 days, the remaining
were bled to death in 20 to 25 days, and all showed hemorrhages
characteristic of scurvy   None of the guinea pigs on sprouted
grain showed scurvy symptoms.

    The alkaline reserve was determined by a modification of the
Van Slyke, Stillman, and Cullen method.   We found their
method satisfactory, but modified it a little so as to compare
more readily our previous data with the present determinations.

Neutral red fades in the standard solutions and we found that dibromothymolsulfonephthalein was more permanent and gave striking color changes, but we did not have enough of it and confined our determinations to neutral red, making new standards every day For standards, we used the borax mixtures of Palitzsch since they are better and cheaper than phosphate mixtures. Since Palitzsch does not show the continuous curve for these mixtures, and we determined ours with the hydrogen electrode, we give the following directions for making them.

We made stock solutions of 0.05 molecular borax and 0.2 molecular boric acid and protected them in resistance glass flasks with automatic burettes with soda-lime tubes. We used reagent borax dried in air as it came from the manufacturer, noting that none of the crystals was moist or had effloresced. We recrystallized the boric acid and dried it in a desiccator. The water was freshly distilled and CO_2-free air was blown through it for 15 minutes. The following table shows the pH and the per cent of the borax stock solution, the remainder being boric acid stock solution.[2]

pH	6 6	6 7	6 8	6 9	7 0	7 1	7 2	7 3	7 4	7 5	7 6	7 7	7 8	7 9
Per cent of borax	2 5	2 7	3 2	3 9	5 0	6 1	7 4	9 9	10 6	12 7	15 1	17 7	20 5	23 7

pH	8 0	8 1	8 2	8 3	8 4	8 5	8 6	8 7	8 8	8 9	9 0	9 1	9 2
Per cent of borax	27 3	30 9	35 2	39 7	44 4	49 3	54 7	60 7	67.4	74 5	81 5	89 2	96 8

We found that the dilution of plasma recommended by Van Slyke is sufficient, as we got practically the same results with double or quadruple that dilution, but that the CO_2 remaining in the plasma after rotation made a difference of about two drops in the titration. This is no objection to Van Slyke's method, but we wished to blow out the CO_2 as thoroughly as we did in the electrometric method in order to compare results by the two methods The flask was rotated by an electric motor while a stream of CO_2-free air was blown through it, as shown in Fig. 3.

[2] We are indebted to Professor Grace Medes for most of the work in preparing the standards

The method may be summarized as follows. Tubes were prepared for the blood by drying in them enough 25 per cent potassium oxalate solution to make 0.2 per cent dry oxalate in the blood.   The animal was anesthetized lightly with ether and the blood was drawn from the left ventricle (carotid in rabbits) and centrifuged immediately.   1 cc. of plasma was introduced in a 100 cc. flask of resistance glass and 20 cc. of distilled water, 0.3 cc. of 0.1 per cent solution of neutral red, and 3 cc. of 0.01 N HCl were added.   Three similar flasks were made up with 25 cc. of
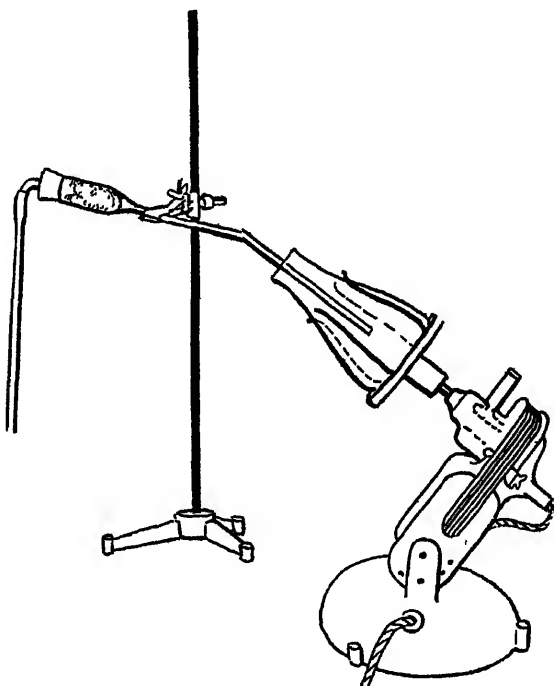
FIG 3.

standard solution and 0.3 cc. of indicator in each, showing pH $= 6.6$, 7.0, and 7 4.   The plasma flask was rotated 5 minutes and, if the color came within range of the standards, more acid was added, but it is not necessary normally to add more acid.   The plasma was then titrated in the same flask with 0.01 normal solution of CO_2-free NaOH to the three standard colors in succession.   Both

acid and alkali were made up with CO_2-free distilled water and
kept in automatic burettes that fill from the top so that the
solutions do not pass through a greased stop-cock before entering
the burette.

The following table shows the alkaline reserve in terms of a
normal solution, as titrated to the three standards.   Owing to
changes in ionization by dilution, we prefer not to say what pH
is the correct end-point, but any end-point may be noted by
drawing a curve through the three points.

No	Scurvy guinea pigs (dry barley)			Controls (barley with 1 in sprouts)		
	pH			pH		
	6 6	7 0	7 4	6 6	7 0	7 4
1	0 026	0 024	0 021	0 016	0 014	0 012
2	0 021	0 019	0 017	0 018	0 016	0 014
3	0 034	0 032	0 026	0 021	0 019	0 017
4	0 020	0 019	0 017	0 020	0 019	0 017
5				0 022	0 021	0 019
6	0 022	0 020	0 017	0 019	0 018	0 016

It may be seen from the table that with the exception of Scurvy
Guinea Pig 3, which was in a moribund condition with dark
venous blood, there is no essential difference between the alkaline
reserve of those with and without scurvy.   If the alkaline reserve
changed during ether anesthesia, and differently in different indi-
viduals, that would account for the slight differences shown, but
not for the similarities of the two groups.   We must conclude
that acidosis has nothing to do with scurvy in the guinea pig.

The weight curves of the rabbits are shown in Fig. 4, those on
sprouted grain are shown by unbroken lines and those on dry grain
by broken lines.   The spaces on the ordinate represent differences
of 300 gm. in weight, and the space on the abscissa represents an
interval of 20 days.   The rabbits were numbered 1 to 3 beginning
with the pair at the top (900 gm.).   The rabbits on sprouted
barley remained nearly constant in weight, while those on dry
barley lost and two of them died before blood was drawn.   The
900 gm. rabbit on dry grain showed scurvy lesions when blood
was drawn on the 21st day.   Before the beginning of the experi-
ment, all the rabbits had been kept together and fed on oats and

hay.   Hence they were particularly susceptible to a scorbutic diet.
The alkaline reserve is shown in the following table.

No	Scurvy rabbits (dry barley)			Controls (barley with 1 in sprouts)		
	pH			pH		
	6 6	7 0	7 4	6 6	7 0	7 4
1	0 008	0 006	0 003	0 009	0 008	0 004
2				0 011	0 010	0 008
3				0 007	0 006	0 004
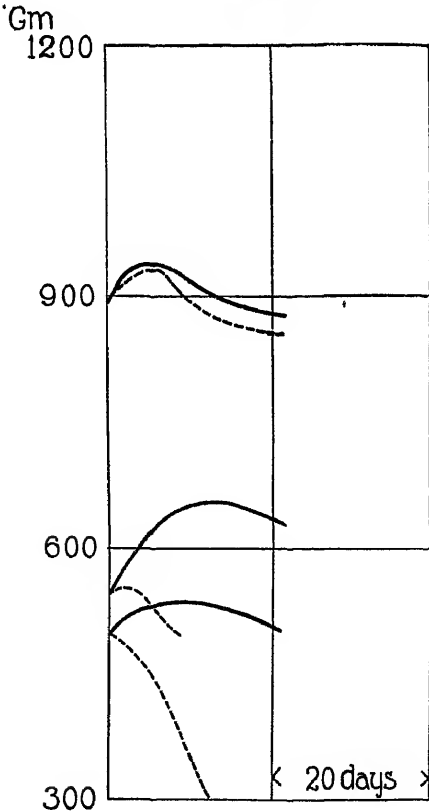
FIG 4

In our previous determinations on rabbits fed on carrots and
hay (McClendon, von Meysenbug, Engstrand, and King), the

alkaline reserve was found to be about 0.020, and hence both sets of the above rabbits show acidosis due to the deficiency of alkali in the exclusive barley diet.   The scurvy rabbit does not show a significant difference in alkaline reserve from the controls, and therefore we may conclude that acidosis has nothing to do with scurvy in rabbits, but may be a factor in the early death of the animals.

According to Fürst, barley sprouted for 3 days prevents scurvy, whereas Weill and Mouriquand found that 10 days sprouting was required.   These workers do not record the temperature, and since the rate of sprouting is more than doubled by a rise of 10° in temperature, it is necessary to control and record the temperature or determine the degree of sprouting by the length of root and acrospire.   In the following experiment an attempt is made to determine the degree of sprouting and quantity of malt necessary to prevent scurvy in the guinea pig, but an epidemic of pneumonia made the data fragmentary.

In this experiment barley seedlings of three ages were used (1 day, 2 days, and 3 days).   In 1 day the roots were $\frac{1}{8}$ inch in length, in 2 days the acrospire was visible, and in 3 days the acrospire projected $\frac{1}{2}$ inch beyond the grain.   The growth curves of the guinea pigs are shown in Fig 5.   Fifteen of these guinea pigs were divided into five sets, numbered 1 to 5, and arranged from left to right (Fig. 5)   The curves of the guinea pigs fed on 1 day sprouts are shown by unbroken lines, those on 2 day sprouts by broken lines, and those on 3 day sprouts by dotted lines.   The divisions on the ordinate represent differences of 100 gm. in weight, and those on the abscissa intervals of 30 days   The first set was fed 1 gm of sprouts, the second set 2 gm , the third set 3 gm., the fourth set 4 gm , and the fifth set 5 gm. per 100 gm of body weight of guinea pig per day, and they had oats and water *ad libitum*.

It may be seen from the curves that nearly all the guinea pigs began to gain in weight and then to lose; the end of the curve represents the early death   Besides this series, one guinea pig, represented by the chain of circles in Fig 5, was fed oats and 2 day sprouted barley *ad libitum* and he ate less than 25 gm. per day of the latter   He died at the end of 33 days with no symptoms of scurvy.   The fact that guinea pigs of this weight die of scurvy

in about 20 days indicates that 2 day sprouts contain an appreciable amount of antiscorbutic substance.   Another guinea pig, represented by the chain of crosses in Fig. 5, was fed oats and 3 day sprouted barley *ad libitum*, and ate less than 25 gm. per day of the latter.   He gained in weight for 32 days.   The decline in weight following was apparently accompanied by an infection that caused death of some of the guinea pigs, but was manifested by a coryza and dyspnea in this one.   At the end of the 48th day we discontinued the oats and gave him the sprouts and autoclaved
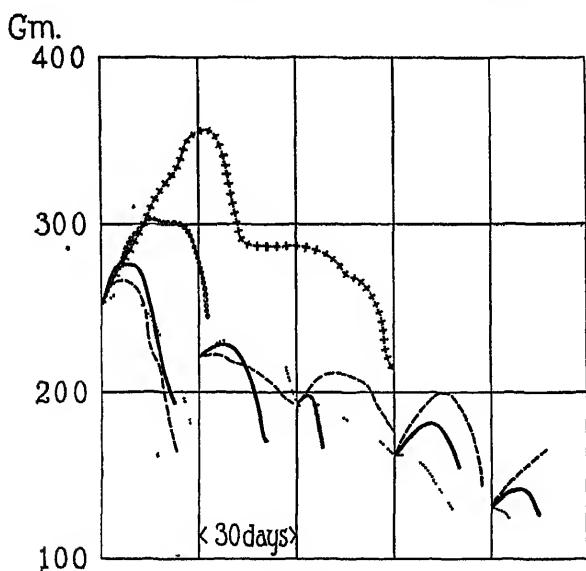
Fig 5

biscuit containing condensed milk for a few days, and then fed him exclusively on sprouted barley and water until he died at the end of 90 days without symptoms of scurvy.   Since barley is deficient in salts, protein, and fat-soluble A, as shown by Steenbock, Kent, and Gross, and salts, at least, cannot be synthesized in the sprouting of the barley, the death of this guinea pig might have been due to lack of salts.

Since the above experiments show that barley, sprouted until the acrospire is ½ inch long or longer, contains considerable antiscorbutic substance (and the same is true of other grains) efforts

were made to prepare sprouted grain for human food and preserve the antiscorbutic substance. The husks of barley make it poor eating, but wheat and rye offer no mechanical difficulties. If sprouted wheat and rye are heated to 70°, the starch is gelatinized and it may be eaten as a salad or breakfast food.   Three guinea pigs were fed exclusively with wheat and rye sprouted until the acrospire projected $\frac{1}{2}$ inch beyond the grain and placed in water of 70° until the starch was gelatinized. Their growth curves are the first three beginning at the left in Fig. 6. The weight is marked on the ordinate and the divisions on the abscissa represent intervals of 30 days. None of these animals showed symptoms of scurvy, but two of them died rather early. The

FIG. 6.

fact that one of them lived 34 days and showed no scurvy symptoms at autopsy shows that the sprouted wheat and rye contain antiscorbutic substances which are not destroyed by heating to 70° to gelatinize the starch. Another guinea pig shown in Fig. 8 was cured of scurvy by a similar diet.

Since the whole grain cannot be fed to infants, we attempted to prepare a juice containing the antiscorbutic substance. Since the antiscorbutic substance probably exists in the cells of the acrospire or roots, and it is difficult to crush these cells, we used a special mill for the purpose, shown in Fig. 7. This mill has polished steel rollers, 2 25 inches in diameter, and differs from an ordinary malt mill in that the rollers are geared to one another and there are scrapers to remove the crushed sprouts. A pulley wheel, 2 feet in diameter, was fitted to one of the rolls and driven

by a belt. Sprouted wheat or rye showed a tendency to slide out
of the groove between the rolls, but sprouted barley fed well into
the mill owing to the roughness of the husk. After the sprouted
barley was crushed between the rolls it was placed in a canvas
bag in a press capable of exerting a pressure of 5,000 pounds to
the square inch It was found, however, that very little juice
came out of it at full pressure and a new bag had to be used each
time. By adding water before pressing, less pressure was sufficient
and the bag lasted longer.
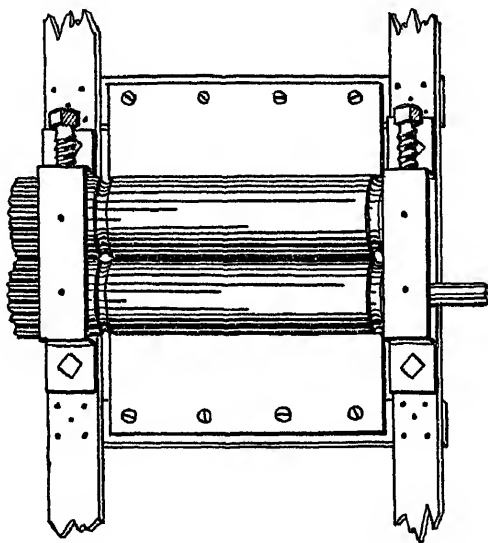
Fig 7.

Three guinea pigs, whose weight curves are shown in the right
half of Fig. 6, were fed with the juice of barley with the acrospire
$\frac{1}{2}$ inch beyond the grain and water *ad libitum*. They almost
maintained their weight, lived about 30 days, and showed no
signs of scurvy at autopsy One guinea pig, shown in Fig. 8, was
cured of scurvy with this juice. This indicates that the juice
contains sufficient antiscorbutic substance. It has a grassy taste,
but probably could be fed to babies without much difficulty.
No doubt the grain could be practically freed from bacteria before
sprouting (Duggar and Davis).

Since very little starch is hydrolyzed during the sprouting and most of it remains in the press, an attempt was made to mash the green malt so that the carbohydrate would appear in the extract. Barley with sprouts $\frac{1}{2}$ inch long (acrospire extending $\frac{1}{2}$ inch beyond the grain) was crushed by passing it through a clothes wringer with rubber rolls, mixed with twice its weight of water, and heated to 70° to gelatinize the starch. It was allowed to remain in the same vessel until the starch iodide reaction disappeared, which required about an hour, and then strained and pressed so that the extract ran into a glass distilling flask with a side neck. A rubber stopper was inserted through which passed a capillary tube extending to the bottom of the flask. The flask was placed in a water bath, heated to 70°, and suction was applied to the side neck so as to evaporate the contents. A fine stream of air bubbles, liberated from the end of the capillary tube, prevented bumping. When the malt extract was evaporated to the consistency of a very thick syrup, i e. the thickest syrup that could easily be removed from the flask, it was stored in glass jars until used A slight amount of oxidase remained in the extract and caused a very slow darkening of the surface exposed to air. Fermentation was prevented by the evaporation, but mould would grow very slowly on the surface if planted there. Some of this extract has been kept for 5 months in good condition.

The weight curves of the guinea pigs fed on this malt extract are shown in Fig. 8. The body weight is marked on the ordinate, and the divisions on the abscissa represent intervals of 25 days The guinea pigs were divided into six pairs which were fed on increasing quantities of extract from left to right, 0, 5, 10, 15, 20, and 25 gm. per guinea pig per day mixed with a dough or mush The mush for one guinea pig consisted of 12 gm of Graham flour, 12 gm of rolled oats, 12 cc of evaporated milk, and 1 cc. of a salt solution containing 25 per cent NaCl and 6 per cent $CaCl_2$, together with the designated quantity of extract. The mixture was a mush only with the larger quantity of extract, and a stiff dough with the smaller quantities. Water was given *ad libitum* as in all the above experiments. All the animals lost weight finally, although they ate greedily until they all showed symptoms of scurvy (tender swollen joints, falling hair, and loose molars) at about the 15th day, after which they ate less The larger guinea pig of the fourth

pair showed marked scurvy on the 16th day and was transferred
to a diet of sprouted wheat and rye heated to 70°, as shown by the
broken line continuation of his curve in Fig. 8.   He gained con-
siderably in weight, and the scurvy symptoms rapidly disappeared
so that none was found at the autopsy on the 26th day.   This
indicates that the process of heating to 70° to gelatinize the starch
does not destroy the antiscorbutic substance.   The reason all the

Gm
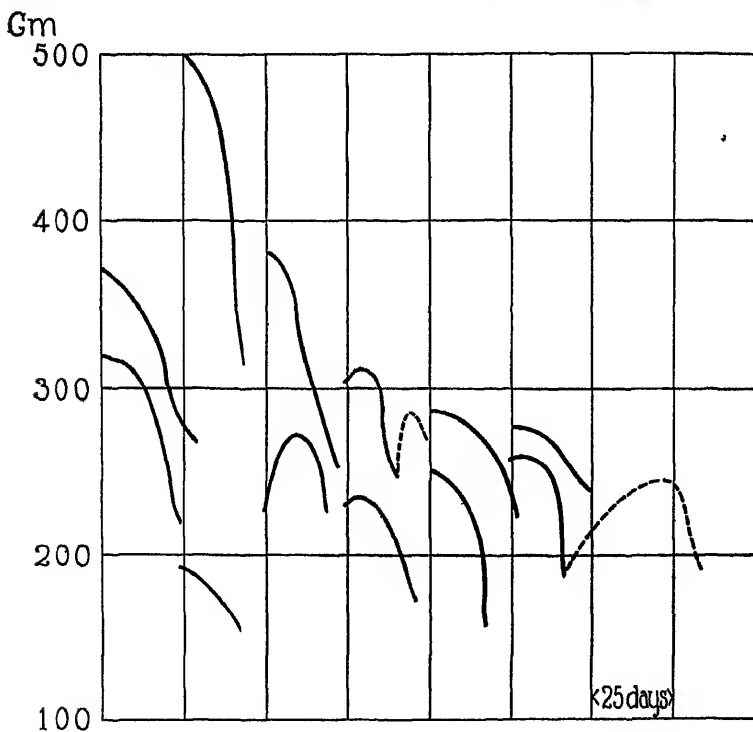
Fig 8

animals developed scurvy may lie in the possibility that the
sprouts were not sufficiently crushed and the antiscorbutic sub-
stance was never extracted from them.   The smaller guinea pig
of the sixth pair developed marked scurvy with swollen wrists
(which when touched provoked a squeal), loose molars, and bloody
diarrhea.   About the 20th day he could scarcely move about his
cage and was changed to a diet of raw juice of sprouted barley
crushed between steel rolls, as shown by the broken line continuing

his curve in Fig. 8.   He gained rapidly in weight and lost all
scurvy symptoms but finally began to lose and died on the 60th
day showing no signs of scurvy on autopsy.

With the exception of the two animals cured of scurvy, the
diagnosis of scurvy in all of the twelve was confirmed on autopsy.
The first pair, receiving no malt extract, lost weight but little more
rapidly than the average of those fed extract.   Since Furst has
shown that commercial malt extract is deficient in antiscorbutic
substance, and the same is true of beer according to Smith, it seems
probable that the only way to get the antiscorbutic substance into
the extract is to crush the green malt between rolls that thoroughly
break up the cells of the acrospire.   It is also desirable to sprout
the grain to a more advanced stage than is done merely for the
development of diastase.

At autopsy some guinea pigs showed impacted cecums, but
this was only in case the animal died of scurvy and never if it
was killed when the scurvy symptoms first appeared.   We
assume that the impacted cecum, as observed by McCollum and
Pitz, is due to the fact that the guinea pig drinks little water
during the last day or so of its life.[3]

<div align="center">CONCLUSIONS.</div>

Acidosis has nothing to do with scurvy.

Sprouted cereal grains, especially after the acrospire projects
$\frac{1}{2}$ inch beyond the grain, are rich in antiscorbutic substance (in
this we merely extend the work of Furst, and Cohen and Mendel).

The antiscorbutic substance in sprouted grain is not destroyed
by heating to 70° to gelatinize the starch.

The antiscorbutic substance may be extracted from sprouted
barley after crushing it between steel rolls that are so close together
that the cells of the acrospire are crushed.   In order to make the
green malt feed between the rolls they must be geared to one
another so as to turn at the same rate.

[3] The keeper of the stock rabbits said he fed one rabbit in a separate
cage exclusively on oats for 9 months   One of us at autopsy of this rabbit
found no marked gross lesions except a fragility of the bones   One rib
had broken spontaneously   Rabbits are much more resistant to scurvy
than are guinea pigs

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TOXICITY OF PHENYLACETIC ACID.

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The retention of protein material in the intestine and its subsequent putrefaction leads to the formation and absorption of many protein decomposition products which are more or less harmful to the organism.

Phenylalanine on putrefaction yields three different aromatic acids, phenylpropionic acid, phenylacetic acid, and benzoic acid, ' while tyrosine undergoes analogous decomposition.

However, if phenylpropionic acid (1) is introduced into the gastrointestinal tract and absorbed, no phenylacetic acid is formed but it is subjected to the process of β-oxidation, loses two C atoms, and is changed directly into benzoic acid.

The benzoic acid combines with glycocoll and is excreted in the urine as hippuric acid. *p*-Hydroxyphenyl propionic acid (1) in a like manner is oxidized to *p*-hydroxybenzoic acid and is excreted as *p*-hydroxyhippuric acid. Phenylacetic acid resists oxidation and remains to be altered by combination with other compounds

Salkowski (2) considered the acid relatively non-toxic and believed that it existed even in normal human urine as the free acid. This was perhaps because he had found only the combined acid in the urine of animals, such as dogs (2, 3), rabbits, and horses and was unable to isolate either the free or combined acid from human urine.

Huppert (4) fed phenylacetic acid to patients suffering from alkaptonuria and proved that the acid aided no way in the formation of homogentisic acid but was unable to find even a trace of the acid after feeding the patient a 10 gm. dose.

Hotter (5), who ingested the acid himself, could find neither the combined or uncombined acid in his urine, so concluded

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that it was oxidized to benzoic acid and excreted as hippuric acid. Phenylacetic acid is particularly interesting from a physiological standpoint on account of its different metabolic action in the organism of man, animal, and fowl.

In the human body, the acid is combined with glutamine and excreted as phenylacetyl glutamine (6). This is the only case so far recorded where the amino-acid glutamine has been used by the body for the purpose of detoxicating a poisonous substance. Animals fed on phenylacetic acid detoxicate it by joining it with glycocoll and excreting it as phenaceturic acid (2).

After feeding the acid to a hen, Totani (7) isolated a compound from the excreta which he terms phenacetornithuric acid. This substance is a combination of one molecule of ornithine with two molecules of phenylacetic acid.

Phenylacetic acid, while found in only small amounts in the · normal human body is one of the most important protein putrefaction products and is by no means as non-toxic as was previously supposed.

A hen weighing 2.23 kilos, after receiving 1 gm. of the acid, refused to eat. A second dose of 1 gm. 3 days later caused the hen to lose weight and develop marked signs of intoxication. A dog weighing 32 6 kilos was able to take 3 gm. of the acid with no apparent signs of discomfort; however, after receiving a dose of 7 gm. of the acid it became very thirsty, refused to eat, seemed to be greatly nauseated, and vomited several times.

A monkey of 4.2 kilos body weight, which received a dose of 1 gm. and refused to eat for several days, also developed a marked diarrhea. Twelve adult humans (male) after ingesting 5 gm. each of the acid showed in every case practically the same symptoms. The sodium salt of the acid was dissolved in 200 to 300 cc. of water and rapidly drunk. In 15 to 30 minutes after the ingestion of the acid, the subject became thirsty and this symptom was rapidly followed by a feeling of hunger. If food was ingested, symptoms of nausea developed; in case no food was taken, a feeling of dizziness resulted, followed either by drowsiness or increased nervousness. One subject weighing 59.1 kilos ingested as much as 16 gm of the acid within a period of 2 hours. Within a few minutes, the usual sensation of dizziness and hunger developed, so the subject partook of an unusually heavy meal and went to bed

1 hour after ingesting the last of the acid, he was unable even to stand unsupported. After sleeping soundly for 6 hours, he awoke and drank 1 liter of water, immediately fell asleep, and again slept soundly for nearly 8 hours. On waking he demanded water and drank more than $1\frac{1}{2}$ liters. He complained of nausea, headache, pain in the eyes, and of loud ringing in the ears   He was able to sit alone but seemed unable to stand unsupported or to correlate his movements. After another 4 hours of sleep, he appeared quite refreshed and normal in every way. In this case there was no sign of diarrhea but on the contrary he presented an obstinate case of constipation, which lasted for about 3 weeks.

In many respects the symptoms of poisoning by this acid resemble those of alcoholic poisoning.

EXPERIMENTAL.

In order to determine the toxicity of the acid, we decided to feed a small dog increasing doses of the substance and to determine if possible the minimum dose which would cause death and to study as carefully as possible any pathological changes produced by the acid.

A small dog of 7 5 kilos body weight was selected and placed in a metabolism cage for observation. The acid was fed to the dog as a water solution of the sodium salt by means of a stomach tube   On the 1st day of the experiment, he received 1 gm. of the acid   On each succeeding day, the dose of the acid was increased by 1 gm

During the first 24 hours of the experiment following the 1 gm dose of the acid, the dog showed no signs of discomfort but ate as usual and showed no signs of abnormal thirst. On the 2nd day after receiving the 2 gm of the acid, the dog showed an abnormal appetite and drank often but only a small amount of water each time. On the 3rd day, he showed signs of drowsiness, but ate as much as usual and drank a large amount of water   On the 4th day, the dog refused to eat, spent most of the time in sleep, and seemed scarcely able to stand when removed from the cage   He was unable to walk and weighed at this time only 6.35 kilos. On the 6th day he still refused to eat and seemed to be in a semicomatose condition.

Up to this time there had been no signs of albumin in the urine but at this point a sufficient quantity was present to give a decided reaction. On the 7th day of the experiment, the dog appeared very weak and after receiving 7 gm. of the acid, underwent a series of convulsions during which time he vomited most of the acid. As much of this acid as possible was reclaimed and weighed. The total amount vomited was approximately 5 5 gm. so the dog received in fact only about 1.5 gm. of the phenylacetic acid on the 7th day.

For about $2\frac{1}{2}$ hours after receiving this last dose of the acid, he appeared quite lifeless, then suddenly underwent a second series of convulsions, which ended in death

Autopsy —Performed about 6 hours after death   Male dog weighing 6 30 kilos   Gross examination of the organs presented no morphological lesions with the exception that the kidneys on section were congested and somewhat swollen, the cortex being pale; medulla congested, capsule non-adherent.   Portions of liver, kidney, spleen, stomach, and alimentary canal were taken for microscopic examination   The specimens were fixed in Orth's fluid and mounted in paraffin.   Sections were cut 6 microns in thickness and examined with $\frac{1}{12}$ oil immersion, ocular 10

Microscopic Examination —The tunica fibrosa of the kidney does not appear to be thickened and the nuclear elements show no deficiency in staining qualities..   The capillaries in the cortex corticis are engorged and the cellular elements within them appear disintegrated.   While there is a general engorgement of the blood vessels, of the cortical portion of the organ, there is no extravasation of blood in the interstitial tissue.

The epithelium of the proximal convoluted tubules is much swollen and granular, so that the lumen of the tubule is in many places completely occluded by the approximation of the distal edge of the epithelial cells Some of the tubules contain in their lumen the remains of broken down epithelial cells but this is in localities distinct from those in which the lumen is occluded and may indicate epithelial areas, which bore the effects of a greater toxic action of the drug   Blood elements are not seen within the tubules

A glistening, hyaline material is found in the lumen of many of the tubules and in the cytoplasm of many cells the same material is seen The engorgement of the capillaries between the tubules is marked   The degeneration of the epithelium is most marked in the proximal convoluted tubules in the neighborhood of the Malphigian corpuscles, becoming less marked as the descending loop is approached

The arched collecting tubules are filled with the hyaline material and then epithelium, including the nuclei, is in many places destroyed   The epithelium of Bowman's capsule is likewise destroyed in many of the

renal coipuscles and while shrinkage of the glomerulus from the capsule is not piesent in every instance, yet it occuis in many of the coipuscles, particularly in those near the boundaiy zone of the medulla, and in the capsular space an exudate of hyaline and granular material and blood cells is present

A round cell infiltration of the stroma of the glomerulus occurs and the capillaries of the tuft are engorged with blood

In the medulla of the kidney, both limbs of Henle's loop show marked destructive changes of their epithelium   This in many places is totally disintegrated, so that the lumen of the tubules is filled with a mass of cellulai remains and misplaced nuclei and a fine reticular mass, staining deeply with eosin, is present   Such epithelial cells as are not destroyed are detached in places from the wall of the tubules

The epithelium of the straight collecting tubules does not appear affected by the action of the diug   All the cells are in place, the nuclei distinct, and the cytoplasm is clear   The nuclear membrane is distinct and the lumen of the tubule, for the most part, empty

The interstitial tissue was not altered in appearance or amount

The microscopic examination of the liver shows the cells to contain a number of globular refiacted spaces, vaiying in size and in some instances occupying the gieater part of the cell   The staining quality of many of the nuclei of the liver cells was markedly deficient   Unfortunately a section was not stained foi fat

The spleen was negative

The stomach and intestines presented nothing of note.

SUMMARY.

The microscopic findings would seem to indicate that as a result of excessive doses of phenylacetic acid in the dog, the secreting epithelium of the pioximal convoluted tube of the kidney is markedly affected; that the endothelium of the blood vessels is not affected, that the epithelium of the arched collecting tubule shows evidence of a destructive action, while that of the straight collecting tubule appears to escape

The secreting epithelium of the limbs of Henle's loop is most distinctly involved, the fact that the interstitial tissue of the kidney is not injured and that the liver changes is in all probability secondary.

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THE CHEMICAL IDENTIFICATION OF THYROXIN.

SECOND PAPER *

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Thyroxin is a white, highly crystalline substance, odorless, and tasteless   It may be separated from aqueous or alcoholic solutions in microscopic crystals which are not soluble in any organic solvent, except those which are strongly basic or acidic in nature.   It is soluble in alcohol in the presence of mineral acid or an alkali metal hydroxide   It is stable toward heat, and its melting point is in the neighborhood of 250°C.   Since it is odorless and colorless and is not easily affected by oxidation and reduction, its most important chemical and physical properties are concerned with the acidic and basic groups within the molecule   Thyroxin is a weak acid, but possesses basic properties in the presence of mineral acids

In 1915, it was suggested that the organic nucleus in thyroxin is indole (1).   Its solubility in alkali metal hydroxides, but not in carbonates, indicated that it was of phenolic nature, and its salt-forming power with acids was attributed to an imino group   After it was known that thyroxin contained about 60 per cent of iodine, and before the empirical and structural formulas were determined, the chemical properties of the molecule were best expressed by di-iodo-di-hydroxy-indole.

The first derivative of thyroxin, which helped to give an insight into its chemical structure, was the sulfate   Thyroxin which was precipitated from alkaline alcohol by acetic acid was found to contain 65 per cent of iodine   Thyroxin, precipitated by boiling an aqueous ammoniacal solution, also contained 65 per cent of iodine   Thyroxin, precipitated by adding sulfuric acid to an aqueous alkaline solution and boiling, was found to contain 60 per cent of iodine   The difference in iodine content was shown to be due to the formation of a salt with sulfuric acid, and by estimating the molecular weight of thyroxin from the molecular weight of

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sulfuric acid, it was found to be 585   With hydrochloric acid substituted for sulfuric, an iodine content, slightly higher than theoretical, indicated that the hydrochloride was hydrolyzed to some
extent   Thyroxin in free form precipitates as needles, but the
hydrochloride separates in flat, rectangular, and star-shaped plates
Examination of the crystals of the hydrochloride, which contained
more iodine than theoretical, showed both the free form and the
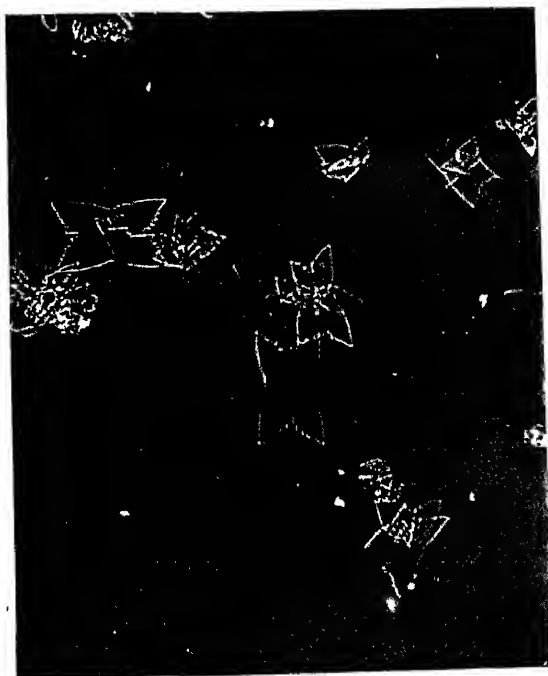
FIG 1 The hydrochloride of thyroxin which separates in flat plates,
rectangular, or star-shaped

hydrochloride.   The sulfate of thyroxin does not hydrolyze with
water so readily as the hydrochloride (Figs 1 and 2)
    Ultimate analysis of thyroxin gave the percentages of carbon,
hydrogen, oxygen, nitrogen, and iodine, and from these and the
molecular weight determination of 586 the empirical formula
was shown to be $C_{11}H_{10}O_8NI_3$ In constructing the structural
formula we were guided by the following.

1 Acidic Properties —Thyroxin is readily soluble in sodium, ammonium, and potassium hydroxide, and is insoluble in sodium, ammonium, and potassium carbonate as ordinarily tested   It is soluble in aqueous sodium and potassium carbonate, however, if very little carbonate is added and the solution boiled.   It is precipitated by carbon dioxide from an alkaline solution   The empirical formula and these acidic properties, therefore, suggest the presence of one carboxyl group, which has very weak acidic properties, and a hydroxy group.

Fig 2 The free form of thyroxin separated as a sheaf of needles.

2 Basic Properties —Thyroxin forms salts with mineral acids, but not with weak organic acids   Thus, together with the fact that thyroxin forms a ureide with cyanic acid, is evidence for the presence of an imino group   The identification of the indole nucleus by the pine-splinter reaction after alkaline fusion was evidence that the imino group was present as in indole   Accepting the presence of the indole nucleus, there remained three extra carbon atoms, a carboxyl group, a hydroxy group, three atoms of iodine, and three extra hydrogen atoms, whose positions in the molecule were to be determined   Since tautomerism is common in the indole group, it seemed probable that the position of the

hydroxy group was adjacent to the imino, and that the three carbon atoms including the terminal carboxyl were attached to No 3 position[1] of the indole nucleus   This structural formula, approximating that of tryptophane, satisfied all that was known concerning the chemical properties of the molecule except the position of the three iodine atoms and the three extra hydrogen atoms.   As no special difference was demonstrable between the reactivity of the three atoms of iodine, it seemed most probable that they were all attached to the benzene ring, and as three extra hydrogen atoms would be required, if the iodine was added to, and not substituted for, hydrogen on the ring, they also were placed on the benzene ring   This formula is a tetra-hydro derivative of indole, the three atoms of iodine being substituted for three of hydrogen on the reduced benzene ring
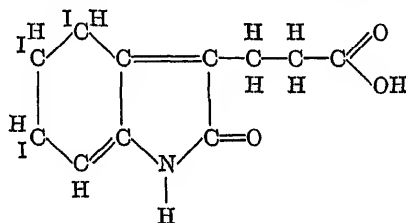
4, 5, 6 tri-hydro-4, 5, 6 tri-iodo,-2 oxy,-beta indolepropionic acid

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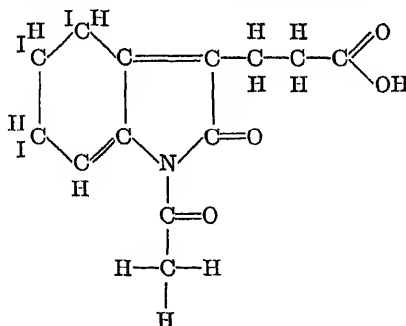
[1] In this paper the positions in the indole nucleus will be referred to as follows.
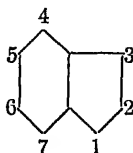
In proving the formula the first derivatives were those involving the imino group   By the addition of acetic anhydride to a slightly alkaline, alcoholic solution of thyroxin, the hydrogen of the imino is replaced with acetyl and the acetyl derivative may be separated in crystalline form by the addition of sulfuric acid and water and the removal of the alcohol by boiling under

Fig 3   The crystal form of the acetyl derivative of thyroxin.

diminished pressure   The sulfate of the acetyl of thyroxin is thus formed   This is dissolved in a small amount of alcohol and when added to boiling water the acetyl of thyroxin separates in pure form (Fig 3)   The melting point of the acetyl is slightly lower than that of thyroxin, it crystallizes in the form of needles more curved and much shorter than those of thyroxin, and although thyroxin is insoluble in all organic solvents the acetyl is readily

soluble in alcohol, ether, ethyl-acetate, and dilute aqueous ammonia and pyridine. The close approximation, by analysis, of the theoretical percentage of iodine in the acetyl, 60 77, corroborates the molecular weight of 585.

Another derivative of the imino group which is easily formed is the ureide.
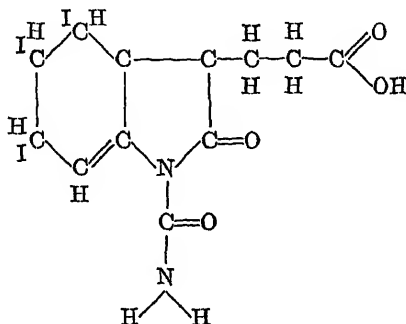
This is made by the addition of a salt of thyroxin, either the sodium or zinc salt, to acetic acid to which potassium cyanate has already been added. Cyanic acid reacts with thyroxin with the formation of the ureide   It separates from boiling water in curved needle form and has very closely the same solubilities as the acetyl (Fig 4). Analysis of the ureide shows the percentage of iodine to agree with the theoretical, 60.67. This is a third confirmation of the molecular weight 585   Although thyroxin forms a stable salt with sulfuric acid which is not hydrolyzed by boiling in dilute sulfuric acid, the addition of the acetyl or ureide groups to the imino increases the acidic properties of the imino, and these derivatives do not form stable salts with sulfuric acid except at low temperatures. Boiling the sulfate in dilute sulfuric acid causes a complete hydrolysis and separation of the acetyl or ureide in free form   The presence of the imino group in thyroxin is established by identification of 'the indole nucleus, the formation of the acetyl and ureide derivatives, and by the power to form salts with mineral acids

The evidence for the carboxyl and hydroxy groups is as follows. Thyroxin is extremely insoluble in aqueous solutions of all acids, including carbonic.   It is very easily soluble in sodium potassium and ammonium hydroxide, but the weakness of the acidic groups

on the molecule is shown by the fact that boiling water alone causes
a complete hydrolysis of the ammonium salt and free thyroxin
may be piecipitated in ciystalline form by boiling an aqueous or
alcoholic solution of its ammonium salt   Dilute solutions of so-
dium and potassium carbonate will dissolve only a small amount
of thyioxin in the cold, but it is soluble in very dilute solutions of
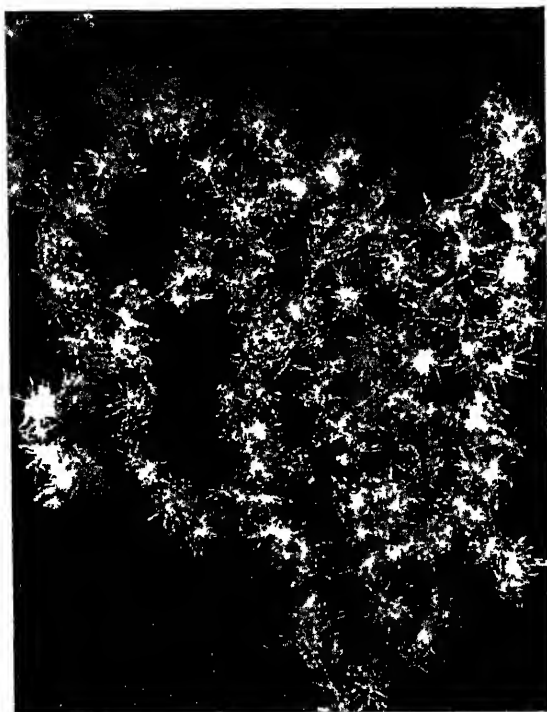
Fig 4 The ciystal foim of the uieide deiivative of thyioxin

sodium and potassium caibonate at 100°C.   Howevei, on cooling
such a solution, a mono-metal salt of thyioxin sepaiates in
crystalline foim   If an excess of carbonate is present at first, the
mono-salt of thyioxin is so insoluble in the piesence of the excess
sodium oi potassium carbonate that most of the thyioxin being
tested iemains insoluble   The addition of a veiy slight amount
of sodium oi potassium hydioxide to a solution containing a

suspension of thyroxin in the presence of sodium or potassium carbonate immediately carries the thyroxin into solution. These reactions suggest that there are present in thyroxin both carboxyl and hydroxy groups. The carboxyl group reacts with carbonates but the resulting mono-salt is so slightly soluble that the presence of excess carbonate forces the mono-salt out of solution. The hydroxy group in the presence of carbonates alone does not react, but the addition of hydroxide to such a solution forms a metal salt with the hydroxy group, and the di-metal salt is readily soluble.

Still further evidence for this action is found in the barium salt Barium chloride added to a sodium hydroxide solution of thyroxin precipitates thyroxin in needle crystals, usually twined, or in sheaves or bundles If this is filtered off it is found to be slightly soluble in boiling water On cooling and with the addition of a soluble barium salt to the solution, the barium salt of thyroxin recrystallizes quantitatively If red litmus paper is dipped into a boiling aqueous suspension of the barium salt, the solution reacts neutral, but wherever the crystals of the barium salt come in contact with the paper the color of the indicator is changed to blue, showing that hydrolysis of the barium salt has occurred. If sodium hydroxide is added to an aqueous suspension of the barium salt of thyroxin the barium salt is dissolved and becomes almost as soluble as the sodium salt This behavior is explained by the fact that the second hydroxyl group of barium hydroxide is not sufficiently strong to form a soluble salt with the hydroxy group of thyroxin In the presence of boiling water the hydroxy group of thyroxin and one hydroxy group of barium exist in free form, barium forming a salt only with the carboxyl group in thyroxin Both hydroxy and carboxyl groups are slowly hydrolyzed by prolonged boiling of the barium salt in water.

The difference between the two acid groups is also shown in a sodium potassium, or ammonium hydroxide solution of thyroxin. If carbon dioxide is bubbled through such a solution so as to produce sodium carbonate, but not bicarbonate, the hydroxy group is freed from metal and the mono-metal salt of thyroxin separates in flat crystals, oval, rectangular, or square If an excess of carbon dioxide is passed through the solution, the carboxyl group also is freed and thyroxin will separate. The sepa-

ration of the monosodium salt occurs at the point where the hydroxy group has been freed, but the carboxyl group is still in the form of a salt (Figs 5 and 6).

While endeavoring to separate the metal salts for analysis the monosodium, potassium, and ammonium salts of thyroxin were prepared by dissolving thyroxin in strong solutions of the hydroxides and passing carbon dioxide through these until the

FIG 5 The mono potassium salt of thyroxin which separates in small flat plates, rectangular, or square

mono-metal salt separated   The crystals were filtered on a small Buchner funnel, and washed with water   It was found that approximately 60 per cent of the amount of thyroxin taken was left on the funnel after drying in the supposed form of the monosodium, potassium, and ammonium salts   It was also found that if the salt was washed on the funnel with a 20 per cent solution of

sodium or potassium chloride, and not with water, the compound
did not melt. When, however, the mono-salt was washed with
water the sodium, potassium, and ammonium salts all had the same
melting point, 204° Since the mono-salt of thyroxin does not
melt and the sodium, potassium, and ammonium salts, washed
with water, all melt at exactly the same point, it seemed probable

Fig 6 The monoammonium salt of thyroxin which separates in long
blades.

that the washing with water was sufficient to hydrolyze the very
weak carboxyl, with the result that free thyroxin was left on the
paper, the base being entirely washed away In order to deter-
mine this the monoammonium salt was prepared as above, filtered,
and washed with water and then analyzed for ammonia by means
of Nesslerization Nesslerization, although an exceedingly sensi-
tive test for ammonia, failed to show the presence of even the

faintest trace of ammonia in the supposed monoammonium salt of thyroxin.   It was therefore evident that by washing the monosodium, ammonium, and potassium salts of thyroxin with water the weak carboxyl group can be completely hydrolyzed, and since the hydroxy group already was in the free form, the molecule existed with both carboxyl and hydroxy groups uncombined with metal
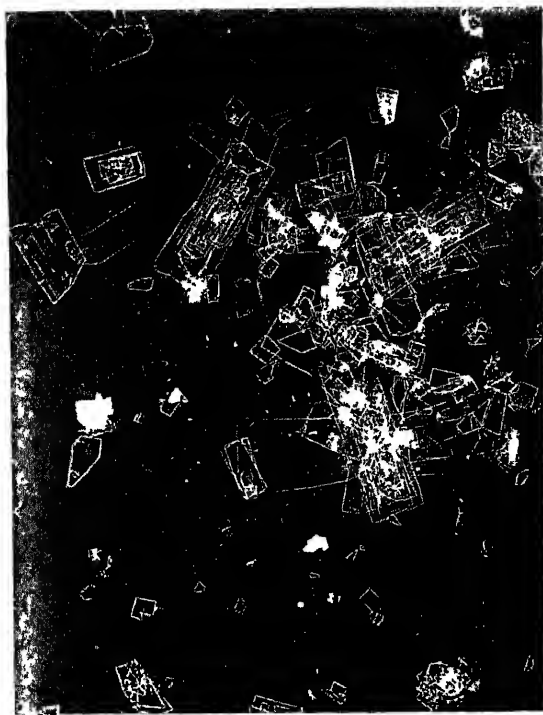
Fig 7 The disilver salt of thyroxin which separates in large, flat, rectangular and square plates, often occurring in sheaf form and twined

Evidence that a di-metal derivative of thyroxin does form is furnished by the silver salt   Although a disilver salt containing the theoretical amount of iodine has not been prepared, this salt has been made with so much silver present that it amounted to 92 per cent of the theoretical for the addition of two atoms of silver to the molecule   The reason why the theoretical disilver salt

cannot be prepared is undoubtedly due to the weakness of the hydroxy group (Fig 7)   When the disilver salt, which is highly crystalline, is washed in order to remove the excess of silver nitrate and ammonia which are used in its formation, the hydroxy group hydrolyzes to some extent, and the amount of silver remaining is slightly less than theoretical

FIG 8 The dipotassium salt of thyroxin which separates in flat plates with rough, irregular edges

⌈ Di-basic salts of sodium, ammonium, and potassium are formed by dissolving thyroxin in the respective hydroxides, and adding a corresponding salt of the alkali preferably the chloride until the di-alkali salt of thyroxin becomes insoluble and precipitates in crystal form (Fig 8)   Di-basic salts, which are only slightly soluble, have also been prepared with barium, calcium, magnesium, nickel, zinc,

and copper (Fig 9)   Although all these salts may be made in beautifully crystalline and characteristic form, it is impossible to filter and separate them in a high state of purity by washing with water   Just as hydrolysis of the hydroxy group caused a lower silver content than theoretical with the silver salt, the hydrolysis of the hydroxy group with the barium salt shows a lower percentage
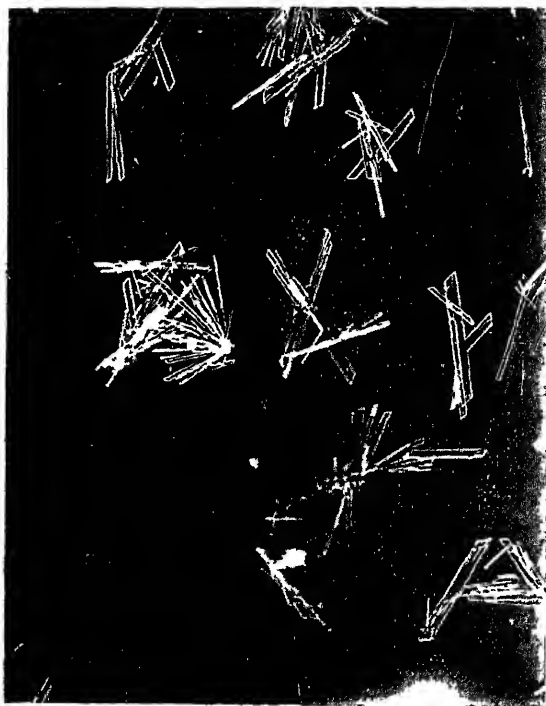
Fig 9 The zinc salt of thyroxin which separates as long, flat blades in bundles and rosettes

of iodine than theoretical.   However, the weight of barium added is about twice that required for the mono-metal salt   With the less basic properties of calcium, magnesium, zinc, nickel, and copper, even greater differences occur between the amount calculated for a di-metal salt and the amount found   But in every case since the amount of metal present nearly agreed with that required

for a di-basic salt, the possibility that these are mono-basic salts is excluded. All salts of thyroxin which are insoluble in water such as the silver, copper, zinc, nickel, calcium, and magnesium are soluble in sodium hydroxide   The solubility is probably due to the same reactions that occur with the barium salt. The weak basic properties of the metals are insufficient to form soluble di-basic salts, but sodium hydroxide carries the salt into solution by adding to the hydroxy group of thyroxin.

Other evidence for the carboxyl and hydroxy groups is furnished by the dimethyl ester   Methyl iodide added to an alcoholic suspension of the silver salt forms the dimethyl ester. This is soluble in alcohol but insoluble in water even in the presence of sodium hydroxide. By heating in dilute alcoholic sodium hydroxide, hydrolysis of the methyl ester of the carboxyl occurs and the oxymethyl derivative is obtained.

Tautomeric Forms of Thyroxin.

Thyroxin reacts in the presence of alkalies forming dibasic salts, but differences between the two acidic groups indicate that one is a carboxyl and one a hydroxy group. When thyroxin exists in this form, which will be called the enol, the hydroxy group is adjacent to the nitrogen but there is a double-bond between the nitrogen and the alpha carbon, and no hydrogen is attached to the nitrogen
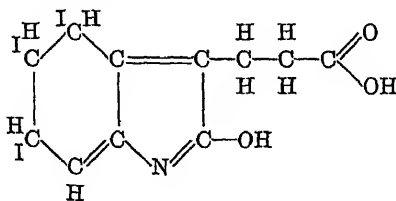
In acid solution thyroxin forms derivatives which demonstrate the presence of an imino group and exists in its tautomeric form, with imino carbonyl groups adjacent   This will be called the keto form. When thyroxin was first isolated it was in the keto form and although it seemed probable that the hydrogen migrated in alkaline solution with a change from carbonyl to hydroxy groups, no quantitative data were available for proof of the hypothesis.

When the mono-metal salts of sodium, ammonium, and potassium were prepared by freeing the hydroxy group in alkaline solution with carbon dioxide, it was found that by washing with water complete hydrolysis of the carboxyl also occurred.   When the hydrolysis of the ammonium salt was carried out at 100° both acidic groups were not only freed but, in addition, the boiling water caused

FIG. 10. The crystals of the enol form of thyroxin

a change from the enol to the keto form   However, if the mono-metal salts are hydrolyzed with cold water the enol form is retained   There are many differences in the chemical properties of the enol and keto forms, but the most striking difference is in the melting point   The melting point of the enol form is 204°, that of the keto, 250°.

When the enol form of thyroxin was prepared by cold hydrolysis of its ammonium salt, it still retained the crystal form of this salt,

but by dissolving the crystals in pyridine and adding water the
enol form separated in its own characteristic crystal form (Fig.
10).   The enol form of thyroxin separates in needle crystals which
are much shorter than those of the keto form and always occur in
rosettes or sheaf-like bundles   Crystallization does not alter the
melting point.

FIG 11. The simultaneous crystallization of both enol and keto forms
of thyroxin from an aqueous pyridine solution

The keto form of thyroxin is by far the more stable and, unless
precautions are observed, the enol form readily passes over into
the keto   The most important factors influencing the change
from enol to keto form are the presence of water and the hydrogen
ion concentration   By adding water to a pyridine solution of the
enol form, conditions may be produced in which both enol and keto
forms simultaneously crystallize (Fig 11)   On long standing even

at room temperature, the enol form slowly changes over to the keto and the keto form alone separates   Since the chief chemical properties of thyroxin are due to its basic and acidic groups, a brief summary of solubilities and reactions of the two tautomeric forms is of interest.

The enol form of thyroxin is much more soluble than the keto, and the solubility may be used as a test of the form in which thyroxin is present.   For example, the keto form is insoluble in all organic solvents, such as all alcohols, ether, chloroform, ethyl acetate, acetone, carbon disulfide, quinoline, pyridine, anhydrous or aqueous, and aniline   The enol form is readily soluble in anhydrous or aqueous pyridine.   Therefore, pyridine alone is not sufficiently basic to change the keto into the enol form, but when this change has been produced pyridine readily dissolves thyroxin. Since ammonium hydroxide in water, alcohol, or pyridine will change the keto form to the enol, but pyridine cannot produce this change, the hydroxyl ion concentration necessary for the conversion from one tautomeric form to the other lies between the basicity of dilute ammonium hydroxide and that of pyridine Since the enol changes to the keto in a boiling aqueous pyridine solution, the acidity necessary for the tautomeric change in this direction lies between the narrow limits of the hydrogen ion concentration of a cold and a boiling aqueous solution of pyridine The limits for the change in tautomeric form are at the same time the limits of solubility for thyroxin in alkaline solution   Thyroxin is soluble in enol form, in pyridine and quinoline, but any higher concentration of hydrogen ion causes the change to the keto form and limits the solubility of thyroxin in alkalies   Thyroxin in the keto form remains insoluble in all organic solvents with hydrogen ion concentrations equal to or less than that of glacial acetic acid. It is soluble in formic acid, but the subsequent addition of water causes thyroxin to separate again   Although acetic acid will not make thyroxin soluble in alcohol the addition of a mineral acid renders thyroxin readily soluble in alcohol   Solubility under these conditions is evidence for the formation of salts with the imino group

The acid and basic properties of thyroxin therefore lie between these two limits (1) The formation of salts with acids through the imino group of the keto form with formic acid, but not acetic,

and (2) the formation of salts with alkalies through both the carboxyl and hydroxy groups with dilute ammonia, but not with pyridine   Lying between these two limits are the formation of mono-metal salts through the carboxyl alone in the presence of carbonates but not bicarbonates, and finally the complete precipitation of thyroxin from the alkali metal salts by carbonic acid or by hydrolysis with water in a boiling ammoniacal solution   The mono-metal salts of thyroxin are but slightly soluble in water but are easily soluble in alcohol.   The solubility in alcohol is due to the fact that although only the carboxyl group is combined with metal, the molecule is in the enol and not the keto form.

The imino group of thyroxin reacts with all acids stronger than and including formic acid, but no acid salt of thyroxin is appreciably soluble in water, and even the sulfate which is the most soluble is only very slightly so   The great insolubility of the keto form of thyroxin is one of the most important factors permitting the isolation of the compound   The insolubility of thyroxin is also of importance in a consideration of its chemical properties. As soon as the proper conditions exist in any solution for the formation of the keto form of thyroxin, the reactive groups are thrown almost completely out of the sphere of reaction by the insolubility of the compound

The keto form of thyroxin is soluble in organic solvents only when some acid is present which is capable of forming an acid salt with the imino group   Thyroxin may be conveniently purified by dissolving either in alkaline alcohol with the addition of acetic acid, or by dissolving in acid alcohol with the addition of sodium acetate   In the presence of acetic acid the imino group does not form a salt and thyroxin precipitates in needle form.

Open-Ring Form of Thyroxin

Since thyroxin in keto form is insoluble in bases weaker than ammonium hydroxide and is insoluble in alcohol in the presence of acids weaker than formic, there is a wide range of hydrogen ion concentration in which pure thyroxin is insoluble   These limits of solubility, however, apply only to pure thyroxin in aqueous and alcoholic solutions.   In the presence of certain substances, changes in the acid and basic groups occur and the solubilities of

thyroxin are materially altered and extended   In the presence of the products resulting from alkaline hydrolysis of the thyroid proteins the solubility of thyroxin is so greatly altered that it is completely soluble in carbonic acid, and even acetic acid produces an incomplete precipitation   Hydrochloric and sulfuric acids precipitate thyroxin under these conditions, but in excess they redissolve a considerable percentage of the total amount present The increased solubility in acids indicates an increase in the strength of the basic groups of the thyroxin molecule.   Although pure thyroxin is practically insoluble in pyridine, sodium carbonate, barium hydroxide, and alcohol, partially purified thyroxin is readily soluble in the presence of all these reagents   The increased solubility in weak alkalies indicates an increase in the strength of the acidic groups in the thyroxin molecule   These changes in the chemical properties of thyroxin are most marked during the early stages of purification while there is a large percentage of impurities present, but that the alteration is due entirely to the impurities is disproved by the fact that both increased acidic and basic properties persist even after the removal of all but a trace of the impurities   Furthermore, the addition of certain substances to a solution of pure thyroxin brings about a similar increase in both basic and acidic properties   The solubility of partially purified thyroxin in weak alkalies is in such striking contrast to the solubility of pure thyroxin that it cannot be explained except by a change in the structure of the molecule other than the two tautomeric forms described above.   The exact nature of this change was suggested by a study of the acetyl

In all derivatives of thyroxin involving the hydrogen of the imino group, it is impossible to make the enol form, as the hydroxy group cannot exist   Because of the absence of the hydroxy group these derivatives should form mono-basic salts through the carboxyl group alone, they should be more insoluble in alkalies, and should form insoluble barium and silver salts   Since acid salts of the imino are soluble in alcohol, acetic acid, and ethyl acetate, it seemed probable that derivatives attached to the imino would also make the molecule soluble in these reagents.   After the acetyl and ureide were prepared in pure form they were found to be easily soluble in alcohol, acetic acid, and ethyl acetate, but instead of being less soluble in alkalies, the acetyl was more soluble and could be held in solution with as weak a base as pyridine alone

The acetyl was not only more soluble in weak organic bases but it also formed a silver salt which was completely soluble in dilute ammonium hydroxide   The silver salt of thyroxin will separate from strong ammonium hydroxide, but the silver salt of the acetyl is so soluble that it is impossible to prepare it in the presence of ammonia   By dissolving the acetyl in pyridine, however, the addition of silver nitrate produces a voluminous precipitate which may be washed, dried, and analyzed   When this was done it was found that the acetyl had formed a di-basic salt with silver. The formation of a di-basic salt by the acetyl indicated the presence within the molecule of another acidic group other than the terminal carboxyl.   The simplest change by which a carboxyl group could be formed would be by introduction of a molecule of water between the imino and carbonyl groups, changing the imino carbonyl groups to amino carboxyl groups, and in the case of the acetyl the amino group is combined with one acetyl radical
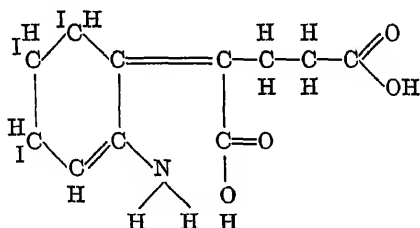
The presence of the acetyl radical in place of the imino hydrogen prevents the tautomeric change to the enol form, but in place of this, the ring opens even in the presence of weak organic bases. The acetyl attached to the amino group, however, does not prevent the closure of the ring, and if an alkaline solution of the acetyl, which is present in the open-ring form, is added to a dilute mineral acid at 100°, the ring closes and the acetyl separates in crystalline form.   In addition to the disilver salt of the acetyl, the zinc salt has been made, and di-basic sodium, ammonium, and potassium salts of the open-ring form of the acetyl may be prepared by dissolving in the respective hydroxides and adding a corresponding salt, the chloride or acetate, until the salt of the acetyl becomes insoluble (Figs 12 to 15)   If sodium hydroxide and sodium acetate are used, very large, flat, jagged plates result;

with sodium hydroxide and sodium chloride short needle crystals are formed

Barium and calcium salts of the acetyl can also be formed by the addition of barium or calcium chloride to a solution of the acetyl in dilute sodium hydroxide or pyridine   An excess of pyridine dissolves the salt

FIG 12 The disodium salt of the acetyl derivative separated from a sodium chloride solution

After the physical and chemical properties of the acetyl derivative had been established, it was found that a most striking resemblance existed between partially purified thyroxin and the acetyl. The acetyl differs from thyroxin in having wider limits of solubility in weak bases, and the greatest difference between partially purified and pure thyroxin is the solubility of the former in weaker alkalies as sodium carbonate, barium hydroxide, pyridine,

and in alcohol   These reactions suggested that in partially purified thyroxin the structure of the molecule is similar to that of the acetyl.   But the possibility that an acid radical was attached to the imino group, as in the acetyl, could be excluded by the fact that the thyroxin could be separated in keto form   The increase in both acidic and basic properties, its close resemblance in chemical
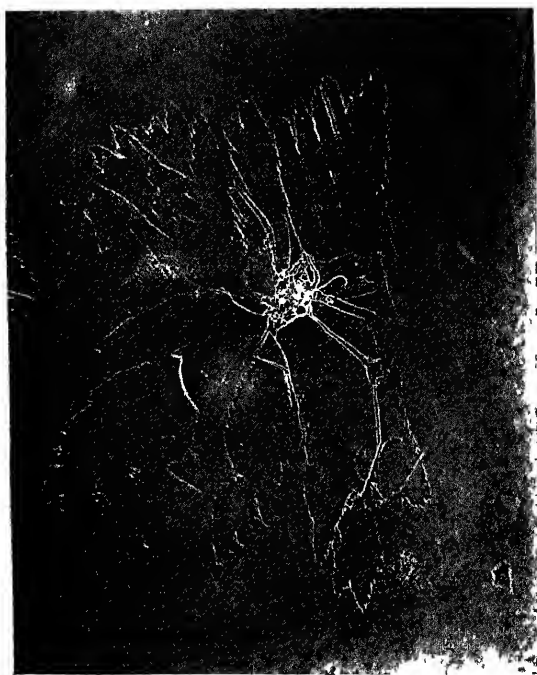
Fig. 13. The disodium salt of the acetyl derivative separated from a sodium acetate solution

reactions to the acetyl, and the fact that it could be separated in the keto form suggest that in partially purified thyroxin the molecule is present neither in the keto nor enol forms, but that the pyrrole ring exists in open form, the elements of water entering between the carbonyl and imino groups

This structure of the molecule of thyroxin will be called the open-ring form.   The open-ring form of indole derivatives con-

taining an alpha carbonyl group is of common occurrence, but thyroxin is perhaps unique in the great ease with which the ring opens and the great difficulty with which the ring closes in the presence of certain substances

Although the open-ring structure of thyroxin was first suggested by a study of the acetyl, further investigation has amply confirmed this hypothesis, and brought to light the delicately balanced

Fig 14   The dipotassium salt of the acetyl derivative

reactions, which, in all probability, are involved when the substance functions physiologically   These reactions are concerned with the opening and closing of the ring and the formation of salts with acids by the amino and imino groups.   When sulfuric acid is added to a slightly alkaline alcoholic solution of thyroxin and the alcohol is distilled, the sulfate of thyroxin separates, the sulfate radical being attached to the imino group   However,

if sulfuric acid is added to an alkaline aqueous solution of thyroxin, the resulting precipitate is not the imino sulfate of thyroxin Analysis of this precipitate for its iodine content showed that thyroxin had not precipitated in free form but contained one equivalent of acid. Further investigation showed that thyroxin precipitates with one equivalent of acid, not only with sulfuric

Fig 15 The dipotassium salt of the ureide of thyroxin which separates in a manner similar to the dipotassium salt of the acetyl derivative

but with weak organic acids and that even carbonic acid adds to thyroxin when carbon dioxide is passed through an alkaline solution The sulfate, chloride, phosphate, trichloracetate, oxalate, formate, acetate, and carbonate of thyroxin have been prepared. All these salts are soluble in alcohol and have melting points which are strangely similar, all of them melting at about 204° Although the imino group of thyroxin in keto form is so feebly

basic that in hot dilute hydrochloric solution the acid radical is hydrolyzed and the imino group exists in free form, when the enol form of thyroxin is precipitated by an acid, an equivalent of acid is contained in the precipitate attached to thyroxin. If any of these salts, prepared by acidifying an alkaline solution of thyroxin, are removed from solution, suspended in distilled water, and boiled, a change occurs, and thyroxin

Fig 16 Crystals of thyroxin in the amino carboxyl form.

precipitates in long, bundle blades. These blades differ from the keto form of thyroxin in being soluble in alcohol and having a melting point of 225°. If instead of suspending the acid salts in distilled water, they are added to a dilute solution of hydrochloric or sulfuric acid and are then boiled, the keto form of thyroxin separates. These reactions are interpreted as follows·

When an acid is added to the enol form of thyroxin, the nitrogen becomes pentad and the acid radical adds to the nitrogen   In

aqueous solution the pyrrole ring is no longer stable and the
elements of water add between the pentad nitrogen and the
carbonyl group forming a carboxyl group and an acid salt of the
amino group
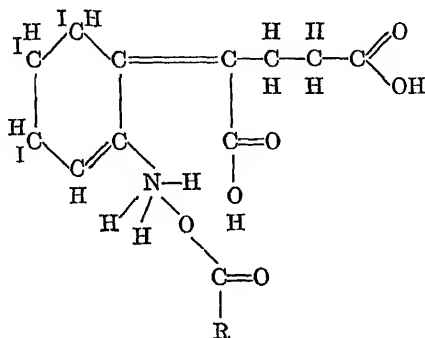
In cold water solution this reaction occurs not only with sulfuric
and strong organic acids, such as trichloracetic and oxalic but
even carbonic acid is capable of adding to the amino group
When the amino salt is suspended in distilled water and boiled,
the amino group is hydrolyzed free from the acid, and the car-
boxyl group which is adjacent to the amino group forms a
salt with the amino group. The compound then exists in an
amino carboxyl salt form, the acid used in precipitating thyroxin
having been expelled from the amino group by hydrolysis with
water.
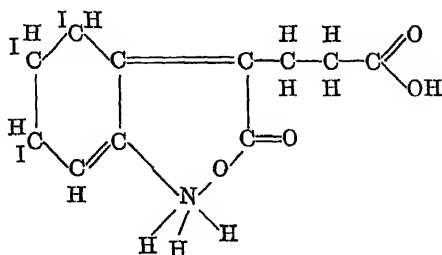
This form of thyroxin differs from both the keto and enol forms
in having the addition of the elements of water   It has a
melting point of 225° and is soluble in alcohol (Fig 16). It is
converted into the keto form very easily, merely solution in alcohol

is sufficient to expel the water, and the keto form of thyroxin then separates   It is impossible to separate the amino carboxyl salt form of thyroxin from solutions containing a high percentage of alcohol   Further investigation showed that this is also true of pyridine and other organic solvents   It is necessary to have water present in order to force the opening of the pyrrole ring

If any acid salt of the amino group of the open-ring form of thyroxin is suspended in distilled water and boiled, the carboxyl

FIG 17 Crystals of the amino carbonate of thyroxin

group in thyroxin, which is adjacent to the amino, will displace the acid radical attached to the amino, and the amino carboxyl salt form of thyroxin results   If the acid radical which is added to the amino is sufficiently strong and an excess of the acid is present, the ring does not remain open, but the elements of water are expelled and the strong acid radical is either hydrolyzed from the imino group or remains attached as an acid salt of the imino group   The closing of the ring of an amino-acid salt is influenced by many factors, such as the amount of acid present, the strength of the acid, and the presence of organic solvents, such as pyridine

or alcohol. Strong acids promptly close the ring, forming imino salts, weak acids are expelled from the amino, and the molecule exists in the amino carboxyl form. The presence of organic solvents such as alcohol results in the closing of the ring and the formation of imino salts with strong acids, or the displacement of the acid with the separation of thyroxin in keto form. If an
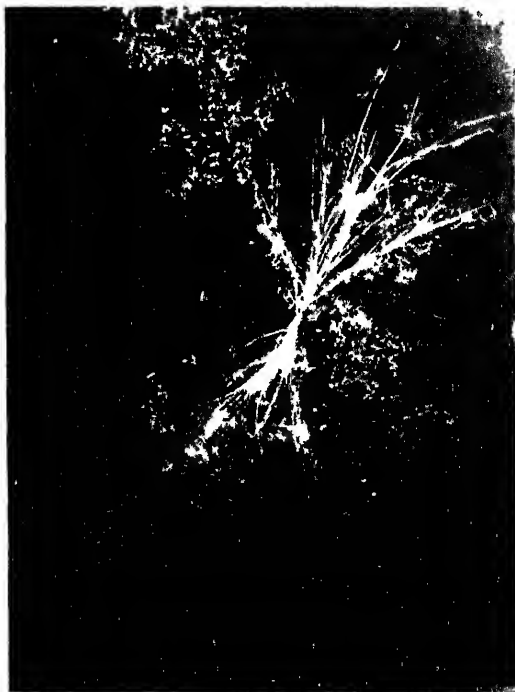
Fig 18 A mixture of the crystals of the amino formate changing into the amino carboxyl salt form of thyroxin   The long needles are the amino carboxyl crystals

amino-acid salt of the open-ring form is washed free from all acid, suspended in neutral water, and boiled, the acid is promptly hydrolyzed from the amino group and the amino carboxyl salt form results, but in the absence of excess acid in solution this form of thyroxin is unstable at 100°C , water is rapidly expelled from the ring, and the keto form of thyroxin separates in very

fine, thread-like crystals.   Under proper conditions all three forms may be present at the same time (Figs. 17 to 19)

Since acids added to the enol form of thyroxin in aqueous solution cause an opening of the ring, the question arose as to whether the opening of the pyrrole ring is the primary action, or whether it is secondary to the existence of the nitrogen in the pentad form   That the opening of the ring occurs without passing

Fig 19 A mixture of amino carboxyl crystals changing into the keto form of thyroxin   The long needles are crystals of the amino carboxyl form of thyroxin

through the enol form is shown by the formation of the amino sulfate directly from the imino sulfate (Figs 20 and 21)   When the imino sulfate is present in a small amount of alcohol and water is added, the ring opens and the amino sulfate separates even though the molecule had existed in the keto form   The formation of amino salts from imino salts shows that the ring opens

readily when the nitrogen is in the pentad state, but that the opening of the ring also occurs directly from the enol form in a solution slightly alkaline may also be shown   When the disodium salt of thyroxin is dissolved in cold water and ammonium chloride is added to the solution, the sodium is hydrolyzed from hydroxy and carboxyl groups, resulting in the precipitation of thyroxin in the enol form   If this suspension of the enol form is now boiled

Fig 20 The imino sulfate of thyroxin separated from a hot solution.

the crystal form changes into the typical amino carboxyl salt form, the melting point of which is 225°   The crystals are also readily soluble in alcohol which excludes the possibility of their being in the keto form   A more direct evidence of the existence of thyroxin in open-ring form is obtained by dissolving the disodium salt in hot water and adding ammonium chloride to this hot solution   Instead of thyroxin separating in the enol form, it

separates directly as the amino carboxyl salt form.   The most important factor in this reaction is the presence of excess alkali. If much alkali is present, the molecule exists in the enol form and addition of ammonium chloride will cause a separation of the monoammonium salt.

From these results it would appear that in acid alcohol solutions thyroxin exists in the keto form   In the presence of excess alkali
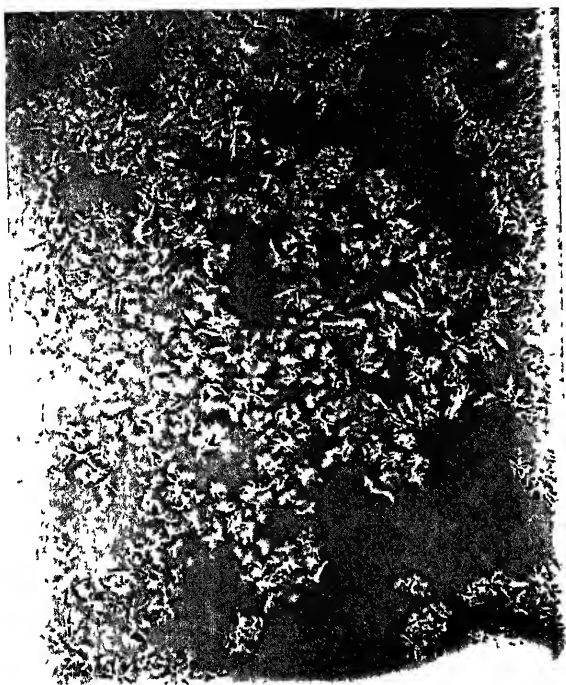
Fig 21 The amino sulfate of thyroxin formed from the imino sulfate of thyroxin by the addition of water to an alcoholic solution of imino sulfate.

in aqueous solution thyroxin exists in the enol form, but as the neutral point is reached from either direction there is a tendency for the ring to open.   In a hot neutral solution the ring does open. In a cold neutral solution even carbonic acid will open the ring and add to the amino group

The Amino Hydrate Form of Thyroxin.

The pyrrole ring of thyroxin not only has a tendency to take up water between the imino and carbonyl groups and exist in amino carboxyl form, but the amino group is so strongly basic that in a slightly alkaline solution the elements of water will add to the nitrogen forming the amino hydrate.
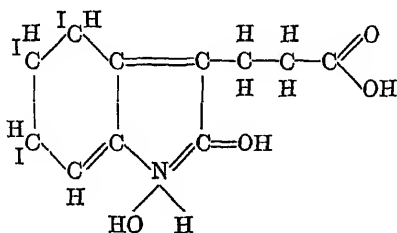
This form of thyroxin is tautomeric with the amino carboxyl form. It is very readily prepared by heating an alkaline solution of thyroxin, removing the solution from the flame, and adding 10 per cent ammonium chloride   The solution becomes turbid, and fine branching crystals separate (Fig 22)   The melting point of this form of thyroxin is 216°   If these crystals are suspended in distilled water containing a small amount of formic acid and the solution is boiled, the crystals are changed into their tauto-meric amino carboxyl form, whose melting point is 225° (Figs. 23 and 24)

One form of thyroxin changes into another so easily that enol and keto forms will crystallize simultaneously out of the same solution, and amino-acid salt, amino carboxyl salt, and keto forms may all be present at the same time, one form changing into another as the boiling of the solution is continued   The ready change of thyroxin from one form to another is explained by the great ease with which the pyrrole ring opens and the elements of water are added to the molecule   This reaction does not occur with indole or isatin and was not at first easily explained in the case of thyroxin   While engaged in the preparation of the inter-mediate products for the synthesis of thyroxin, our attention was drawn to the fact that the explanation of the peculiar properties of the imino group in the pyrrole ring of thyroxin is the presence

of the hydro groups in the benzene ring   Analine is a feeble base
and the imino group in indole is still more feebly basic.   Hexa-
hydro-analine has such a strongly basic amino group that it will
combine with carbon dioxide from the air, and it has a very caustic
action on the skin   It is the addition, therefore, of four hydro
groups to the molecule that so modifies the nucleus giving basic
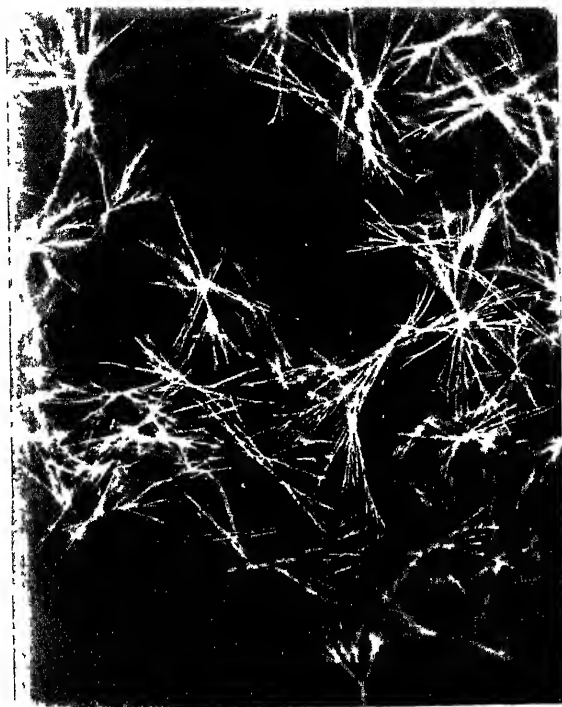
Fig 22 The crystals of the amino hydrate form of thyroxin

properties to the imino group of the pyrrole ring of thyroxin
Speculation as to the properties of the compound in which the
three iodines are replaced by three hydrogens may be deferred
until the substance is prepared synthetically, but that the imino
group of the pyrrole ring will be still more basic in this compound
would naturally follow from the general law that addition of
halogen to the benzene ring renders the ring more acidic.   The

position and reason for the three extra hydrogens in thyroxin were unknown and were very puzzling until the reactions of the compound involving the amino and imino groups caused the necessity of explaining this action by some modification of the indole nucleus.   Since the introduction of the six hydro groups in analine greatly increases the basicity of the amino group, the addition of four hydro groups to the indole nucleus of thyroxin is an adequate

Fig. 23 A mixture of enol and keto forms of thyroxin crystallizing simultaneously from the same solution

explanation of the increased basicity of its amino group   The instability of the pyrrole ring of thyroxin in contrast to that of indole, and other unreduced derivatives of pyrrole is due to the increased basic properties of the nitrogen in thyroxin.   This point is well illustrated in the stability of the amino carboxyl form.   In neutral solution the nitrogen tends to become triad, the pyrrole

ring is more stable than the amino carboxyl salt, and thyroxin separates in keto form   If a slight amount of acid is present, the nitrogen remains in the pentad state and the amino carboxyl form is so stable that it is impossible to expel water from the molecule and make the keto form   The difference in the basicity of the nitrogen when changing from the open- to the closed-ring forms is probably involved when thyroxin functions physio-

Fig 24 A mixture of the crystals of the amino carboxyl form changing into the keto form   The small rosettes are crystals of thyroxin in the keto form.

logically   But the unique chemical properties of thyroxin are also due in large measure to the carbonyl group adjacent to the imino, and the reactivity of the substance *in vivo* and *in vitro* is due to the presence of this oxy group in the indole nucleus.   It was for this reason that the compound was named thyr-oxy-indole or thyroxin

After it was found that thyroxin forms amino salts with feeble carboxyl groups, it was of especial interest to form the amino salt of thyroxin with glycine   Reserving a study of the reaction between thyroxin and the amino-acids for a further communication, merely the formation of an amino-acid salt between thyroxin and glycine will be reported in this paper

Since acid added to the enol form of thyroxin results in the formation of an amino salt, it seemed probable that at least a portion of the nitrogen of thyroxin should react as amino nitrogen with nitrous acid when the molecule existed in the amino-acid salt form   This was tried and it was found that when an alkaline aqueous solution of thyroxin was added to a Van Slyke amino-acid apparatus, approximately 70 per cent of the total nitrogen present was liberated as amino nitrogen   When the keto form of thyroxin was used, no nitrogen was evolved. When the amino carboxyl form of thyroxin is added to nitrous acid, about 15 per cent of its total nitrogen is evolved as amino nitrogen   The reason that a quantitative evolution of amino nitrogen does not occur with the last mentioned form in 3 minutes is because the crystals are insoluble and the reaction takes place at a very slow rate

When nitrous acid is added to an alcoholic solution or to an aqueous suspension of thyroxin in the presence of hydrochloric acid, a yellow color is produced. Upon the addition of ammonia this is changed to a deep red which in dilute solution is pink   This color reaction is convenient for a rough qualitative test for thyroxin. However, if acetic or sulfuric acid is used in place of hydrochloric, a fainter yellow color is produced, and the addition of ammonia gives a yellowish orange instead of a red color

During the purification of thyroxin, the presence of colloidal impurities is sufficient to cause the opening of the ring and also to prevent the closing of the ring   When an alkaline solution of thyroxin is acidified the precipitate carries down many of the impurities present as salts of the amino group and hence no quantitative separation can be effected by precipitation with an acid   The chief problem in the isolation of thyroxin is to close the ring in the presence of the impurities, and thereby produce chemical properties specific to the thyroxin molecule, which permit of a separation   This difficulty in closing the open-ring form of thyroxin is well illustrated in the course of its purification. Approxi-

mately 50 per cent of the iodine content in the early steps of the separation of thyroxin is soluble in barium hydroxide. This barium-soluble portion may be hydrolyzed by heating with barium hydroxide for many hours, precipitated with acid, given another treatment with barium hydroxide, and this process continued as many as seven or eight times without rendering thyroxin insoluble in barium hydroxide. This treatment, however, slowly separates many of the impurities and the percentage of iodine in the dry material may reach as high as 58 per cent. Thyroxin in this open-ring form contains sufficient impurities to impart a distinctly yellow color, and it is readily soluble in sodium carbonate, barium hydroxide, pyridine, and alcohol. By chance such a preparation was dissolved in sodium carbonate solution and was allowed to stand 7 weeks. At the end of that time a white residue had separated and settled to the bottom of the flask. Examination showed this to be the monosodium salt of thyroxin. Although the material was in the open-ring form when dissolved in the carbonate, on long standing the ring had closed and the compound thereby became insoluble in sodium carbonate.

The open-ring form of thyroxin cannot be precipitated from alcohol with acetic acid. The keto form of thyroxin is very nearly quantitatively precipitated from alcohol by acetic acid, but as long as impurities are present, an alcoholic solution of the open-ring form of thyroxin may be allowed to stand several weeks without any trace of thyroxin separating. If, however, an alcoholic solution of thyroxin is slowly evaporated on the water bath, the evaporation causes a partial separation. A yellow oily tar creeps up the inclined bottom of the evaporating dish and forms a ring as the alcohol evaporates. At the spot where the last trace of alcohol was left, a dry crusty material, which is almost white, shows the partial separation of thyroxin in the keto form. This property of thyroxin to separate from alcohol, even in the presence of impurities, is the reaction by which thyroxin was first isolated. The alcohol in this case was evaporated unintentionally, and, although the entire sample of thyroxin had been completely soluble in the alcohol, the slow evaporation and subsequent heating at 100° was sufficient to close the ring in a small percentage of the total amount with the result that it was insoluble on the addition of more alcohol. This method of sepa-

ration is not of great value for the isolation of the compound.
The best method so far determined for the closing of the ring is
to dissolve thyroxin in alcohol containing sodium hydroxide,
and pass carbon dioxide through the solution, freeing both hy-
droxy and carboxyl groups Most of the sodium carbonate is
insoluble and is removed by filtration The alcohol is distilled,
leaving an aqueous sodium carbonate solution of thyroxin but
still in open-ring form Allowing this to stand for several days
will cause a separation of the monosodium salt in the enol form
which may be purified by similar treatment.

Oxidation and Reduction of Thyroxin.

Quantitative oxidation and reduction experiments with thy-
roxin have not been carried out because of the amount of material
which would be required in order to isolate the products
Thyroxin is more susceptible to reduction than to oxida-
tion. Zinc in alkaline or acid solution breaks off iodine and
appears to alter the organic nucleus. Thyroxin is reduced when
heated in the presence of any metal in alkaline solution other
than nickel and the heavy metals, silver, gold, and platinum.
Thyroxin is stable in the presence of mild oxidizing agents Hy-
drogen peroxide produces no immediate effect and in a cold acid
suspension the molecule will resist oxidation with potassium
dichromate or iodic acid. Potassium permanganate or bromine
in hot aqueous solution causes the breaking down of the molecule.
Benedict's copper solution, for the determination of sugar, causes
an oxidation of thyroxin in the presence of sodium hydroxide.
In the presence of ammonium hydroxide alone, thyroxin is stable
in Benedict's solution heated to boiling. Free iodine if added
to an alkaline solution produces a precipitate and apparently
brings about a deep-seated reaction within the molecule. In
acetic acid or acid alcohol, iodine has very little, if any, effect
on thyroxin even at the temperature of boiling acetic acid
Further investigation showed that, in the presence of iodine,
thyroxin is stable in the keto form, but not in the enol form
The changes produced by oxidation with chlorine, bromine, and
iodine, have not been determined, but 'n alkaline solutions yellow
tarry products are formed. Since the enol form of thyroxin is so

much, less stable than the keto, the weakness in the molecule appears to be in the linkage of the nitrogen, and the point of cleavage is probably between the nitrogen and the hydroxy groups. When the imino carbonyl groups are present, the molecule is much more resistant to oxidation by halogen.

Another point of weakness within the molecule exists in the benzene ring. The completely reduced benzene ring readily passes over into a six carbon, straight chain form. Hexa-hydro-phenol may be readily oxidized to adipic acid by the action of dilute nitric acid. In thyroxin the benzene ring is in the tetra-hydro form, and it is highly probable that the one double-bond which is present will break down with the formation of an open chain structure. A reaction similar to this is found in the oxidation of the tetra-hydro-benzene ring of sedanonic acid (2) to straight chain acids.

Due to the weakness of the linkages to the nitrogen in thyroxin, it is impossible to hydrolyze derivatives from the imino group. After the acetyl radical has been attached to the imino group, it cannot be hydrolyzed with sodium or potassium hydroxide. When treated with alkali, the compound precipitates as a di-metal salt, and is thrown out of solution. If sufficiently drastic action is applied to bring about the hydrolysis, disruption of the molecule occurs.

Thyroxin upon exposure to the sunlight in weak alkaline solution is very unstable. Within 24 hours the solution changes from colorless to a pink, or faint yellow, which deepens on standing to a brown color, depending on the amount of thyroxin present. Simultaneously with the discoloration a distinct aromatic odor is produced slightly resembling that of nicotine. Such a solution, when tested for iodine by means of starch in acid solution, shows that no iodine has been broken off in the free form. If, however, a small amount of potassium iodide is added, iodine is immediately liberated, which indicates that the iodine within the thyroxin molecule was not broken off either as hydriodic acid or as iodine, but in the form of hypoiodous acid. On longer standing a test for free iodine is given without the addition of potassium iodide and the amount of hypoiodous acid is much reduced. After several weeks no test for iodine or hypoiodous acid is given, but all the iodine is found in the form of hydriodic

acid. The reduction of the hypoiodous acid to hydriodic acid is probably brought about by the hydroindole nucleus. The finding that iodine is broken off from the thyroxin molecule in the form of hypoiodous acid and not as hydriodic has direct bearing on the physiologic action of the molecule within the body.

The Acetyl Derivative.

With the acetyl, sunlight produces a similar reaction, but in this case the solution is found to contain both hypoiodous acid and iodine. The acetyl derivative, therefore, is more susceptible to oxidation than thyroxin, and brings about a much more rapid reduction of the hypoiodous acid to iodine and hydriodic acid

So unstable is the acetyl under certain conditions that there is a spontaneous liberation of iodine from the molecule and the simultaneous oxidation of the organic nucleus, resulting in a change of color and the production of a yellow tarry material   The conditions under which it is produced appear to be in a solution of approximately the neutrality of distilled water. If barium or calcium chloride is added to a sodium hydroxide solution of the acetyl, and the solution is boiled, no decomposition of the barium or calcium salts occurs. If magnesium chloride is used in place of barium or calcium, the basicity of magnesium hydroxide is insufficient to prevent the decomposition of the acetyl, and there is a spontaneous liberation of iodine accompanied with production of a blue color, which changes to green, and finally to yellow. This same reaction occurs if the sodium salt of the acetyl is dissolved in distilled water and allowed to stand without the addition of sodium hydroxide. Also if the disodium salt is filtered on a small Buchner funnel, washed with sodium chloride, and allowed to stand in a moist condition there is rapid liberation of iodine and production of bluish green colors, which fade to yellow. If small pieces of the acetyl in dry form are added to water, alcohol, or pyridine containing alkali, the solution of the solid material is accompanied by decomposition in part with a liberation of iodine and discoloration. If the acetyl is dissolved in alcohol the addition of sodium hydroxide to the solution will form the sodium salt of the acetyl without decomposition or liberation of iodine. Furthermore, an alkaline solution can be added to acid with precipi-

tation of the acetyl without decomposition. In both acid and alkaline solutions the acetyl is as stable as thyroxin, but at the neutral point a spontaneous decomposition occurs. One of the factors which affects this reaction is the mass of material present. In dilute solutions the decomposition of the acetyl is very much slower, and in sufficiently dilute solutions it may not occur at all. This effect of the mass of material explains why solution in alcohol prevents the destruction of the acetyl with addition of alkali. The mechanism is essentially the diminution of the concentration of the acetyl. Great difficulty was encountered in the preparation of the acetyl until the factors influencing the decomposition were discovered. As thyroxin does not react in this way no difficulty was anticipated, and it was only after identification of free iodine in the solutions of the acetyl which had turned bluish green that an insight into the mechanism was obtained. Why the acetyl derivative spontaneously decomposes at the neutral point and gives off iodine in the free form instead of hypoiodous acid, as occurs with thyroxin, is not known.

The reactions resulting in the oxidation of the acetyl and liberation of iodine are also given by the ureide under similar conditions. This excludes the possibility that the acetyl radical is necessary for the decomposition and suggests that the reason for the instability is the replacing of the imino hydrogen by a larger group

When this decomposition has occurred in part, the products cannot be removed from the rest of the material, and it is impossible to separate the acetyl in free form. The tarry products resulting from the decomposition of a small part prevent the crystallization of the rest of the acetyl. The retention of impurities by the acetyl is very similar to the retaining of impurities by partially purified thyroxin.

Beside the spontaneous decomposition, other reactions which are specific to the acetyl were found which will be discussed at this time. It was found that when the acetyl was freshly precipitated from an alkaline solution by an acid it is soluble in ether. After it has been separated in crystalline form and dried it is insoluble in ether This difference in solubility is undoubtedly due to the acetyl existing in open-ring form when precipitated from cold water solution. The closed-ring form is insoluble in ether.

When the acetyl derivative is prepared by adding acetic anhydride to an alkaline alcoholic solution of thyroxin, and the alcoholic solution of the acetyl is then added to ether the acetyl is removed only partially by subsequent extraction of the ether with sodium hydroxide. A large amount of the acetyl remains in the ether. If the ether solution is tested with nitrous acid the usual reaction with the production of a yellow color turning to red with the addition of ammonia, does not occur. After alcoholic sodium hydroxide is added and the solution is heated, a typical reaction with nitrous acid will take place. Another difference of the acetyl before and after the treatment of the ether solution with alkali is shown in the solubility of the acetyl in alkalies. If the ether is evaporated before alkaline hydrolysis, the acetyl is found to be very difficultly soluble in aqueous sodium hydroxide. After hydrolysis in alkaline alcohol, the acetyl is very easily soluble in dilute alkali. The non-reactivity with nitrous acid and insolubility in alkali may be due either to the formation of an inner salt between the carboxyl and imino, or to the formation of a diacetyl derivative. Treatment with alkalies hydrolyzes either the acid salt or one acetyl group, and the acetyl in free form is liberated.

The Action of Sunlight on Thyroxin, and the Production of Colored Compounds from Thyroxin.

When thyroxin, the acetyl, or the ureide is dissolved in dilute sodium hydroxide and allowed to stand in the sunlight, the solution slowly changes, and in the course of 12 to 72 hours develops a distinctly pink color. On further standing the pink color is changed to yellow. When the carbonic acid derivative was prepared by treating thyroxin with phosgene, it was found to be unstable and changed to a deep pink. When the sodium or barium salt of thyroxin is allowed to stand exposed to sunlight in a dry form, it also develops a pink color. With the barium salt this action does not occur in the dark, or when the salt is covered with water. The development of the pink color in each case is accompanied by the splitting off of iodine in the form of hypoiodous acid. This pink color was first noticed on the edges and outside of white porcelain casseroles which were used to extract

the barium salt of thyroxin with sodium hydroxide   Where the solution dried and was exposed to heat and light the pink color developed   Later this was shown to be due to the thyroxin itself and not to the impurities present.   The chemical structure of the pink-colored compound is still unknown, but it appears probable that it is an oxidation product of the hydroindole nucleus   The effect of light on the separation of thyroxin is of importance, and loss of thyroxin due to the action of light may amount to a considerable percentage of the total unless precautions are observed not to permit the action of direct sunlight to destroy the partially purified thyroxin.

Effect of Acid and Alkali on Thyroxin.

No quantitative determinations have been made as yet concerning the ultimate products of alkaline hydrolysis because of the amount required to isolate the decomposition products   Thyroxin is not affected at room temperature by any concentration of aqueous sodium hydroxide   It is soluble in dilute alkali and after the concentration has reached 10 to 15 per cent the disodium salt separates   The further addition of alkali renders the sodium salt more insoluble but it does not cause any destruction of thyroxin.   Although thyroxin is stable in sodium hydroxide at room temperature, when it is heated above 110° in the presence of strong sodium hydroxide, there is a destruction of the molecule with the splitting off of sodium iodide, and eventually the liberation of indole which may be identified by the pine-splinter reaction   The amount of indole liberated is not quantitative and it is probable that only traces of the hydroindole nucleus appear as indole

In acids thyroxin is not so stable as in the presence of alkali In aqueous solutions of pure thyroxin, hydrochloric or sulfuric acids precipitate the hydrochloride or sulfate of thyroxin, and, since these are insoluble, the destruction of thyroxin is prevented. However, when thyroxin is present in open-ring form, strong acids bring about reactions with the impurities and oily tarry products result   In alcohol solutions prolonged action of hydrochloric acid causes a destruction in part even with pure thyroxin, resulting in the production of a brown discoloration   Poly-

merization of indole compounds in the presence of acid is well established, and it seems probable that this explains the destructive action of acids on thyroxin

A Consideration of Other Possible Structural Formulas

Accepting the empirical formula as $C_{11}H_{10}O_3NI_3$, the carbonyl group in the molecule could be present as a ketone, either attached to the side chain or to the benzene ring.   That this is not the case is shown by the failure of thyroxin to react with hydrazine, phenylhydrazine, or semicarbazone   The carbonyl group adjacent to the imino should not react with hydrazine and the failure of thyroxin to react is evidence corroborating the hypothesis that the carbonyl group is adjacent to the imino   The positions of the three iodine atoms, the three extra hydrogen atoms, and the three carbon atoms in excess of the indole nucleus have not been determined by substitution or by decomposition products   The most conclusive proof of the position of the three carbon atoms and the terminal carboxyl would be furnished by the synthesis of thyroxin   The synthesis of thyroxin will be reported in another paper, but at this time the synthesis of the compound will be cited as evidence for the correctness of the structural formula assigned in regard to the position of the three carbon atoms with terminal carboxyl   The establishment of the fact that thyroxin does not rotate polarized light excludes an asymmetric carbon atom and confirms the arrangement of the double-bonds.

The Crystal Form and Melting Point of Thyroxin

The keto form of thyroxin crystallizes in six distinctly different forms (Figs 25 to 30)   Each of the seven other forms of thyroxin has characteristic crystal forms   The di-basic and monobasic metal salts and the amino- and imino acid salts of thyroxin also have characteristic forms.

The crystal forms of imino acid salts and of di- and mono-metal salts are flat plates for the most part   The other crystal forms are long branching needle blades or thread-like needles which occur in rosettes, or in sheaves, or in tangled masses

Beside the type of crystal, all these forms and derivatives of thyroxin also have characteristic melting points   For these reasons microscopic study of the crystals and the determination of the melting point are the two methods which have proved of greatest value during this investigation.   The determination of iodine is not of such value because the iodine content of any one
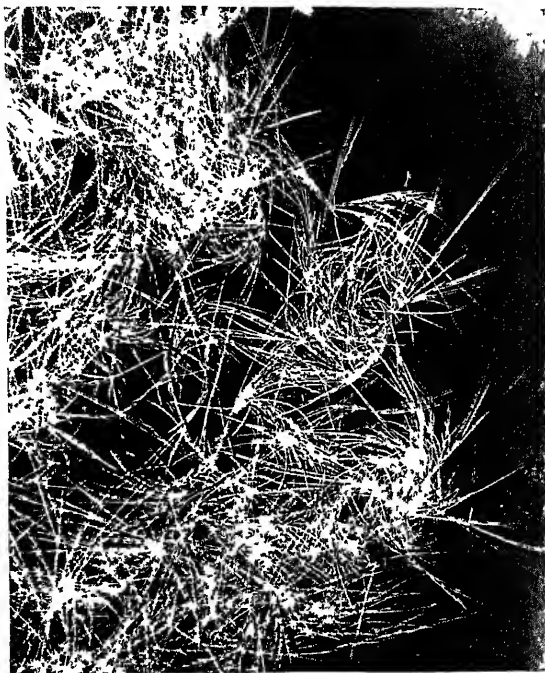

Fig. 25.

Figs 25 to 30   Six different types of crystals in which the keto form of thyroxin separates

form may vary within wide limits without greatly affecting either the melting point or the crystal form.   A good illustration of this is in the keto form of thyroxin   The keto form of thyroxin when pure melts at 250°, when slightly impure the melting point drops to 246° or 245°, and when grossly contaminated with impurities it drops to 240°.   This point, however, appears to be the limiting value below which the keto form seldom melts   It appears that

merely dissolving thyroxin in alkali and precipitating it causes a slight decomposition. The decomposition products are retained by the pure thyroxin and are separated with great difficulty. However, the presence of these impurities rarely exceeds the amount which lowers the melting point to the neighborhood of 240°. Microscopically the keto type of crystals could be identi-

Fig 26

fied, and the melting point of 240° or above would confirm the form in which thyroxin existed but the iodine content might vary as much as 2 5 per cent from the theoretical   The keto form melts the highest of all forms or derivatives of thyroxin, from 240–250°, the amino carboxyl form melts in the neighborhood of 225°, the amino hydrate melts at 216°, and the enol form at 204°; the imino acid salts melt at about 228°, the amino-acid

salts in the neighborhood of 204°; the derivatives of thyroxin attached to the imino, in closed-ring form, melt at about 238° and in open-ring form at about 152°.   It is apparent that the melting points of different forms and derivatives are so widely separated that no misinterpretation could result except in cases of mixtures, and the crystal form is so definite that mixtures can
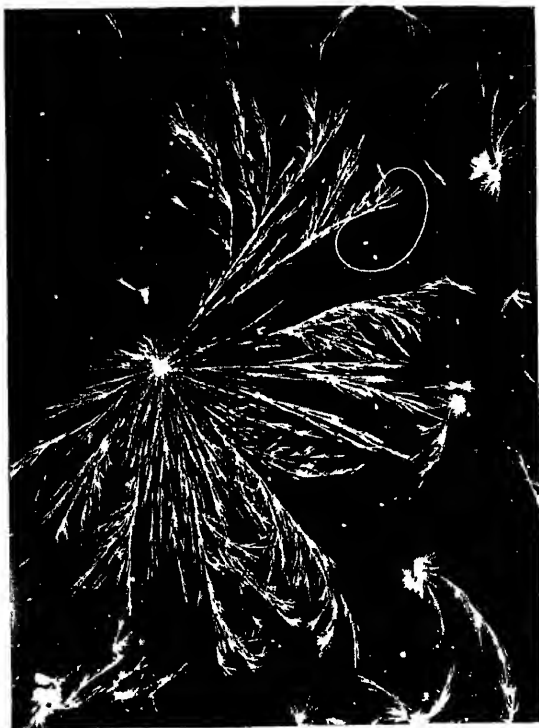
FIG 27

be identified under the microscope.   It has been found, however, that the melting point varies greatly with the rate of heating. One sample of thyroxin, which melted at 240° when heated at the rate of 10° increase per minute, melted at 248° when heated at the rate of 18° increase per minute, and at 221° when heated at the rate of 0 6° increase per minute.
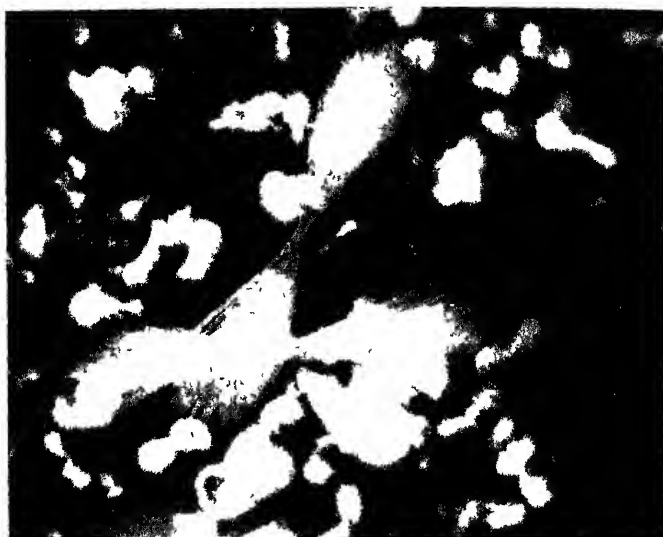
FIG. 28

FIG 29

For a routine determination we have adopted the rate of 10° increase per minute, and when the melting point is observed under these conditions each form of thyroxin and its derivatives agree very closely in their melting points, with other samples of the same form
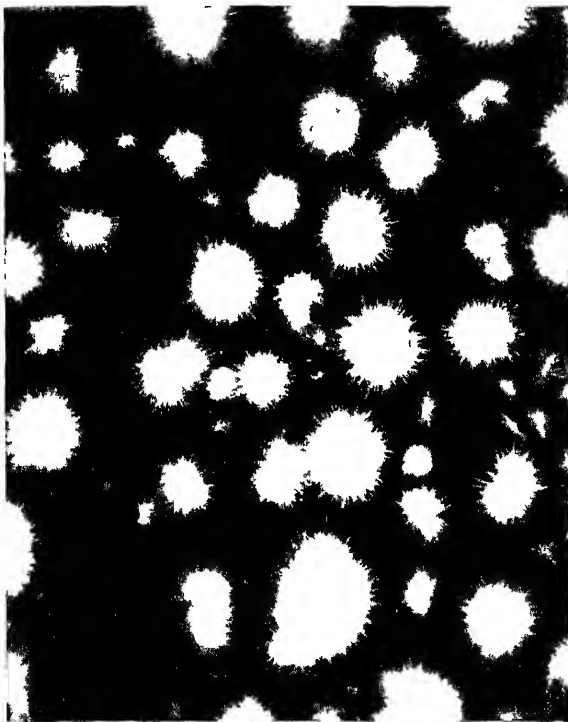
FIG 30

EXPERIMENTAL.

The Percentage of Iodine in Thyroxin

Eighteen different samples of thyroxin have been prepared, the weights of the samples ranging from 900 mg. to 5 gm   All the samples contained at least 63 5 per cent of iodine and six

were purified until they contained approximately 65 per cent of iodine.   The iodine content[2] of Samples 1 to 6 is as follows

Iodine Content of Thyroxin [2]

Sample 1	5 10 mg	contained	3 31 mg. of iodine	= 64 81 per cent.
" 2	5 12 "	"	3.32 " "	" = 64 86 " "
" 3	5 24 "	"	3 41 " "	" = 65 02 " "
" 4	5 10 "	"	3 32 " "	" = 65 00 " "
" 5	25 3 "	"	16 45 " "	" = 65 00 " "
" 6.	4 51 "	"	2 66 " "	" = 65 02 " "

Precipitation of thyroxin from alkaline alcohol with acetic acid, or by boiling an ammoniacal solution, does not vary the percentage of iodine.   Precipitated with acetic acid from alkaline alcohol the iodine content of thyroxin was 64.95 per cent.   Precipitation of the same preparation by boiling an ammoniacal solution gave an iodine content of 64 92 per cent   50 mg. of thyroxin were dissolved in 200 cc. of water containing 3 cc. of concentrated ammonium hydroxide.   The solution was boiled down to 100 cc.   100 cc of water were added and the solution was boiled for a few minutes, cooled, and filtered   15 cc of filtrate contained 0.20 mg. of iodine.

Solubility = one part in 48,800.

Ultimate Analysis of Thyroxin.

Sample No	Weight of sample	CO_2	H_2O	Carbon	Hydrogen	Iodine
	mg	mg	mg	per cent	per cent	per cent
7	201 4	175 2	32 3	23 72	1 78	64 95
7	171 2	148 0	26 9	23 57	1 74	64 95
8	291 0	248 8	45 7	23 32	1 74	64 92
9	124 2	101 9	18 5	22 37	1 65	65 02

102 mg of thyroxin contained 2 27 mg of nitrogen = 2 23 per cent

	Carbon	Hydrogen	Oxygen	Nitrogen	Iodine
	per cent	per cent	per cent	per cent	per cent
Calculated for $C_{11}H_{10}O_3NI_3$	22 56	1 70	8 20	2 39	65 10
Found	22 37	1 65	8 73	2 23	65 02

[2] The iodine was determined by a method published in 1914, which has been modified recently so that it is now applicable for the determination of small amounts of iodine to a high degree of accuracy (The determination of iodine in connection with studies in thyroid activity, Kendall, E C , *J Biol Chem*, 1914, xix, 251)

Solubility of Keto Form of Thyroxin.

50 mg. of thyroxin were dissolved in 200 cc. of dilute sodium hydroxide. 20 cc. of 50 per cent hydrochloric acid were added and the solution was boiled; 15 cc. of the filtrate contained 0.116 mg. of iodine.

Solubility = one part in 84,000.

The Sulfate of Thyroxin.—For the preparation of the sulfate 50 mg. of thyroxin are dissolved in 200 cc. of water containing 50 to 100 mg. of sodium hydroxide; 20 cc. of 50 per cent sulfuric acid are added, and the solution is boiled. The sulfate of thyroxin is soluble at 100°, but on cooling settles to the bottom of the container in oval-shaped plates. It may be filtered on a Buchner funnel, washed with water, and dried in a desiccator.

Analysis of the Sulfate of Thyroxin

Sample 1   5 48 mg   contained   3 27 mg of iodine = 59.71 per cent.
"   2 25 00 "   "   15 00 "   "   "   = 60 00 "   "
114 4 mg of thyroxin sulfate gave 86 9 mg of carbon dioxide, 18 1 mg. of water; 5 08 mg contained 3 04 mg of iodine.

	Carbon	Hydrogen	Iodine
	per cent	*per cent*	*per cent*
Calculated for $C_{11} H_{11} O_5 NI_3 S_{\frac{1}{2}}$.	20 82	1.73	60 09
Found . ..	20 71	1 75	59 92

50 mg. of thyroxin were dissolved in 200 cc. of sodium hydroxide precipitated with 20 cc of 50 per cent sulfuric acid, and the solution was boiled, 15 cc. of the cooled filtrate contained 0.127 mg. of iodine.

Solubility = one part in 76,900

The Hydrochloride of Thyroxin —The hydrochloride of thyroxin is best prepared by dissolving a small amount of thyroxin, 15 to 25 mg in 5 or 6 cc. of alcohol containing 2 to 3 cc of 50 per cent hydrochloric acid   1 to 2 cc of water are added, and the alcohol is boiled off in a test-tube. After most of the alcohol has been distilled the hydrochloride will separate in flat, glistening plates. If too small an amount of acid is used, or too much water, the hydrochloride will hydrolyze, and the plates will change to needles.

The Metal Salts of Thyroxin.—Dry crystalline thyroxin is readily soluble in sodium and potassium hydroxides in the cold, if the concentration of the alkali is less than 10 to 15 per cent. Thyroxin is insoluble in sodium or potassium hydroxide in the cold if the alkali is stronger than 15 to 20 per cent, but it is more soluble in strong alkali if the solution is warmed. In a mixture of 66 per cent of alcohol and 33 per cent of water containing 1 per cent of sodium hydroxide, thyroxin may be dissolved to the extent of about 4 per cent. It is still more soluble in hot solutions but on cooling will separate as the di-metal salt. Thyroxin is not readily soluble in dilute ammonia, but concentrated ammonia will dissolve about 1.8 per cent of thyroxin.

The Di-Basic Sodium and Potassium Salts.—100 mg of thyroxin are dissolved in 20 cc. of dilute sodium hydroxide and to this are added 150 cc. of a solution of 10 per cent sodium hydroxide containing 20 per cent of sodium chloride. If a precipitate occurs the solution is warmed and the clear solution is then allowed to stand until cold. The exact concentrations of sodium chloride and hydroxide are not important. The disodium salt readily separates in any solution containing a high concentration of sodium salts. If the strongly alkaline solution is decanted and replaced with 15 per cent sodium chloride solution, the crystals may be filtered through a Buchner funnel on paper. They are insoluble in 10 to 15 per cent sodium chloride and may be washed and dried. The disodium salt cannot be prepared in pure form for analysis as washing with water dissolves the salt, and it passes through the paper. The di-basic potassium salt is prepared in a way similar to that used for the sodium salt. The diammonium salt is prepared by dissolving thyroxin in hot concentrated ammonium hydroxide and allowing the solution to cool. Flat, rectangular crystals of the diammonium salt will separate.

The Alkaline Earth Salts of Thyroxin.—The barium, calcium, and magnesium salts of thyroxin are prepared by dissolving 50 to 100 mg. of thyroxin in 100 cc. of dilute sodium hydroxide using as small an amount of alkali as possible to carry the thyroxin into solution. The solution is heated to boiling and 20 cc. of a 20 per cent solution of barium, calcium, or magnesium chloride are added to the hot solution of thyroxin. Carbon dioxide is excluded by placing the beaker in an atmosphere free of carbon

dioxide. The magnesium and calcium salts are very slightly soluble in boiling water. 500 cc of boiling water will dissolve between 100 and 150 mg of the barium salt   The barium salt may be suspended in boiling water and the solution filtered through a Buchner funnel into a suction flask.   30 cc of 20 per cent barium chloride are now added to the filtrate and the barium salt is allowed to recrystallize   Only a negligible amount of thyroxin is soluble in the presence of this amount of barium chloride   The barium salt under these conditions is unstable and will slowly turn a pink color.   Analysis for iodine shows a lower iodine content than that calculated.

Iodine Content of Barium Salt

Sample 1. 6 76 mg contained 3 26 mg of iodine = 48 51 per cent
"   2. 5 98 "   "   3 00 "   "   " = 50 24 "   "
Calculated for $BaC_{11}H_8O_3NI_3$:   52 91 "   "

If the barium salt is filtered and dried there is a slow decomposition and the color of the salt becomes yellowish gray.

Silver, Copper, Nickel, and Zinc Salts of Thyroxin —The silver, copper, nickel, and zinc salts are prepared by dissolving 50 to 100 mg. of thyroxin in 25 to 50 cc of concentrated ammonia.   25 cc. of a 10 per cent solution of silver nitrate, copper sulfate, nickel sulfate, or zinc sulfate are made ammoniacal with strong ammonia so that the precipitated hydroxide is just carried into solution, and there is a slight excess of ammonia.   The solution of the metal is now added to the ammoniacal solution of thyroxin, and after standing a short time the crystals of the metal salt will separate.   If too much ammonia is used, a larger amount of the solution of the metal may be required to start the crystallization.   Allowing it to stand over night will insure a more complete precipitation.   Sodium hydroxide may be used for the solution of thyroxin, but, since the metal salt of thyroxin is more soluble in sodium hydroxide, ammonium hydroxide has been found to be the most satisfactory solution   The silver, copper, nickel, or zinc salts suspended in water or dilute ammonia are soluble on the addition of several cc. of 30 per cent sodium hydroxide solution.

Iodine Content of Silver Salt.

Sample 1	4 86 mg	contained 2	37 mg	of iodine	= 48 74 per cent.		
"	2 4 96 "	"	2 44 "	"	"	= 49 15 "	"
"	3 4 00 "	"	1 97 "	"	"	= 49 34 "	"
"	4. 4 06 "	"	1 99 "	"	"	= 49 10 "	"
"	5 4 54 "	"	2 23 "	"	"	= 49 20 "	"
"	6 4 08 "	"	1 96 "	"	"	= 48 00 "	"
"	7 6 02 "	"	2 93 "	"	"	= 48 60 "	"
"	8 4 38 "	"	2 09 "	"	"	= 47 73 "	"
Calculated for $Ag_2C_{11}H_8O_3NI_3$						47 74 "	"
" " $AgC_{11}H_9O_3NI_3$·						55 06 "	"

The average of these eight determinations is 48.73 per cent iodine. The iodine content indicates the addition of 92 per cent ' of the theoretical amount for a di-metal salt and 184 per cent of the amount required for a mono-metal salt. The higher iodine content than that calculated for a di-metal salt is undoubtedly due to the hydrolysis of the hydroxy group. In the zinc salt, the amount of iodine was found to be 56 95 per cent. The calculated amount for the zinc salt is 58.79 per cent, but the calculated amount of iodine in the zinc salt in which hydrolysis of the hydroxy group had occurred would be 57.12 per cent. The close agreement between the amount found and the latter figure is evidence that hydrolysis of the hydroxy group also occurs in the zinc salt of thyroxin. The low iodine content of the salts of the diad metals, barium and zinc, and the high iodine content' of the salt of the monad metal, silver, suggest that in all di-metal salts of thyroxin hydrolysis of the hydroxy group is brought about by washing the salt with water.

Preparation of Mono-Metal Salts. —100 mg of thyroxin are dissolved in 150 cc. of 1 per cent sodium hydroxide, and carbon dioxide is bubbled through the solution until thyroxin is precipitated. The suspension is now heated until solution is complete. On cooling, the monosodium salt separates in crystal form. The separation of the mono-salt is assisted by the presence of sodium salts and is hindered by too great a dilution. An excess of carbon dioxide must not be passed through the solution, as free thyroxin will be precipitated. The preparation of the potassium salt is similar to that of the sodium salt. In preparing the ammonium salt, strong ammonium hydroxide is much better than dilute, as

the solubility of the ammonium salt is thereby decreased. If the mono-metal salt is filtered on a Buchner funnel and washed with 10 to 15 per cent sodium, ammonium, or potassium chloride, it is not dissolved through the paper. If washed with cold water, about 40 per cent is dissolved and 60 per cent of·the amount of thyroxin taken remains on the paper in the form of free thyroxin in the enol form

Nesslerization of the Hydrolyzed Monoammonium Salt of Thyroxin

50 mg. of the residue left on the paper after washing the mono-ammonium salt with water were dissolved in 60 cc. of ammonia-free water containing a small amount of sodium hydroxide. The addition of 15 cc of Nessler's solution and dilution to 100 cc produced no color, showing hydrolysis of the monoammonium salt was complete.

Solubility of Monosodium Salt.—5 cc. of 0.10 per cent sodium carbonate dissolved about 15 mg of dry thyroxin in keto form, when the solution was heated to boiling. Most of the thyroxin reprecipitated on cooling to 20°, but not as the monosodium salt. The crystals were needles or very small rosettes   1 cc. of the filtrate contained 0.76 mg of iodine = 1.17 mg of thyroxin.

Solubility = one part in 850.

5 cc. of 1 per cent sodium carbonate dissolved 85 to 90 mg. of thyroxin at almost the boiling point. Probably more than this amount could be dissolved but as the solution began to turn yellow the heating was stopped. On cooling to 23°, needles in clusters and square plates of monosodium salt separated   1 cc. of filtrate contained 1.14 mg. of iodine = 1.75 mg. of thyroxin.

Solubility = one part in 570.

The Enol Form of Thyroxin.—The enol form of thyroxin is the most difficult to prepare in crystalline form, but is very easily prepared in the crystal form of the mono-metal salts by cold water hydrolysis of the monoammonium, sodium, or potassium salts. 10 to 20 mg. of the enol form in dry powder is readily soluble in 1 to 2 cc of pyridine. The addition of 10 to 15 cc. of water will produce a cloudiness. On long standing (24 hours), the thyroxin will precipitate   Depending on conditions of the con-centration of pyridine and the amount of thyroxin present,

the thyroxin may precipitate in keto form. This may be distinguished under the microscope as long bundles, or sheaves of needles. The enol form separates in either rosettes, or short bundles of needles. The enol form of thyroxin is also soluble in quinoline. Other ways to prepare the enol form of thyroxin is to add ammonium chloride to a cold solution of the disodium salt in water which does not contain an excess of alkali, or to dissolve the disodium salt of thyroxin in alcohol and add water and ammonium chloride. A mixture of both keto and enol crystals usually results if much alcohol is present.

10 to 15 mg of dry crystals of the keto form of thyroxin may be boiled with 5 to 10 cc. of pyridine without going into solution. The addition of a very small amount of dilute ammonia will change the thyroxin to the enol form and carry it into solution. The water may then be boiled off and the enol form remains soluble in pyridine   If 15 to 20 mg. of the enol form of thyroxin are dissolved in 2 to 3 cc. of pyridine in a test-tube, and to this 10 to 15 cc. of water are added, and the solution is boiled, after the pyridine has been removed by distillation, thyroxin will separate in the keto form similar to the precipitation of thyroxin from a boiling ammoniacal solution

25 mg. of thyroxin added in the keto form to 10 cc. of water in the presence of 1 gm. of sodium carbonate will not dissolve. If alcohol is used instead of water and the alcohol is boiled, the sodium carbonate does not dissolve but the thyroxin is carried into solution. The monosodium salt of thyroxin in the enol form produced by the alcoholic suspension of sodium carbonate is readily soluble in alcohol.

The Amino-Acid Salt Form of Thyroxin.—For the preparation of amino-acid salts, thyroxin is dissolved in a small amount of sodium hydroxide in a large volume of water, and to this carbon dioxide or other organic acid is added until thyroxin precipitates. The voluminous precipitate is filtered, washed, and dried. In order to prepare the amino salts in crystalline form, other expedients may be used. For the preparation of the amino sulfate, thyroxin is dissolved either in formic acid or in alcohol containing a small amount of sulfuric acid   The alcohol is concentrated to small volume, and water is quickly added in large volume.

Solubility of Thyroxin in Amino Salt Form

Hydrochloride—50 mg. of thyroxin were dissolved in 200 cc of dilute sodium hydroxide, and precipitated at 25°C. with a slight excess of hydrochloric acid, 15 cc of the filtrate, after removing the precipitate of thyroxin, contained 0 037 mg of iodine

Solubility = one part in 263,000.

Carbonate.—50 mg. of thyroxin were dissolved in 200 cc. of dilute sodium hydroxide, and precipitated by passing carbon dioxide through the solution, 15 cc of the filtrate contained 0.012 mg. of iodine.

Solubility = one part in 815,000.

Iodine Content of Amino Carbonate

5 8 mg contained 3 137 mg of iodine = 60 57 per cent
Calculated for $C_{11}H_{12}O_4NI_3(H_2CO_3)_2$ = 60 09 " "

The slightly higher figure for iodine than the calculated amount is probably due to separation of the thyroxin in either the enol or amino hydrate form, or to the hydrolysis of the amino carbonate and formation of the amino carboxyl. This sample of thyroxin contained 65 02 per cent of iodine when precipitated in keto form

The Amino Carboxyl Form of Thyroxin—The amino carboxyl form is prepared by suspending the amino-acid salt form of thyroxin in water in the presence of a small amount of weak organic acid, such as acetic, and boiling the solution. The acid radical is expelled from the amino group, and the adjacent carboxyl group forms an amino carboxyl salt It can also be prepared by dissolving the disodium salt of thyroxin in a large volume of water, heating to boiling, adding ammonium chloride, and continuing the boiling. The amino carboxyl form of thyroxin separates.

If no acid is present the elements of water may be expelled from the molecule and thyroxin will separate in keto form.

Iodine Content of Hydrolyzed Amino-Acid Salts

A sample of amino carbonate was suspended in neutral distilled water and the solution was boiled 3 minutes. This was not sufficiently long to complete the hydrolysis.

5.38 mg contained 3.321 mg. of iodine = 61.72 per cent.

The same sample boiled 15 minutes showed that all of the carbonate had been expelled and also that some of the amino carboxyl form had been converted into the keto form.

5.2 mg. contained 3.305 mg. of iodine = 63.56 per cent.

A sample of amino sulfate suspended in distilled water and boiled more nearly approximated the amino carboxyl form, but in this case all the sulfate radical was not hydrolyzed.

5 0 mg contained 3 135 mg of iodine = 62 72 per cent
Calculated for amino carbonate, 60 09 per cent iodine
"   "   "   sulfate,   58 43 "   "   "
"   "   "   carboxyl,   63 18 "   "   "

Preparation of the Amino Hydrate Form —Thyroxin is dissolved in a large volume of water with a moderate excess of sodium hydroxide. The solution is heated to boiling and then removed from the flame, and 10 per cent ammonium chloride is slowly added to the amount of 10 to 15 cc. The solution becomes turbid, and long branching crystals separate. The limits of the concentration of the hydrogen ion and the temperature for the formation of the enol, amino hydrate, amino carboxyl, and the keto forms are very narrow. If thyroxin is dissolved in a few mg. of sodium hydroxide, and the solution is diluted to about 400 cc., and divided into four equal parts, each of the four different forms of thyroxin may be prepared from these solutions merely by varying the conditions of the precipitation. Ammonium chloride added to one of the solutions in the cold, will precipitate the thyroxin in the enol form, or as the monoammonium salt in flat plates. If ammonium chloride is added to the second solution, which has been heated to boiling, and then removed from the flame, the amino hydrate form will separate. If the amino hydrate is precipitated in the third, and the solution containing a suspension of the amino hydrate is heated to boiling and the boiling continued, the crystals will change into the amino carboxyl form. If a large excess of ammonium chloride is added to the fourth solution, and the solution is boiled, thyroxin will separate in the keto form.

Open-Ring Form in the Presence of Impurities.—The percentage of iodine in the open-ring form of thyroxin, which is still soluble

in pyridine, sodium carbonate, barium hydroxide, and alcohol, may be between 50 and 60 per cent.

Iodine Content of Thyroxin Still in Open-Ring Form

Sample 1 5 00 mg. contained 2 51 mg of iodine = 50 28 per cent
"   2 3 92 "   "   2 13 "   "   "   = 54 21 "   "
"   3 6 44 "   "   3 51 "   "   "   = 54 46 "   "
"   4 4 16 "   "   2 43 "   "   "   = 58 53 "   "

The solubility of these samples in alcohol, sodium carbonate, and barium hydroxide showed that although the amount of impurities present was very small, the ring still existed in open form.

Colloidal Substances Producing the Open-Ring Form of Thyroxin.— 50 to 100 mg. of pure thyroxin, added to the impurities which are separated during the process of purification, will become readily soluble in alcohol, sodium carbonate, and barium hydroxide, showing the change from the keto to the open-ring form. Gelatin and proteins of blood produce the same changes, but the amino-acids resulting from the hydrolysis of gelatin will not change the solubility of thyroxin in barium hydroxide, alcohol, or sodium carbonate.

The Acetyl Derivative.

Preparation of the Acetyl.—In the preparation of the acetyl it is necessary to use pure thyroxin. The presence of even a small amount of impurities makes it impossible to crystallize the acetyl and it will separate only as an oily tar. 100 mg. of pure thyroxin are added to 20 cc. of alcohol containing 100 mg. of sodium hydroxide. After the thyroxin is entirely dissolved 2 cc. of acetic anhydride are added. The solution is allowed to stand for 30 minutes, 5 cc. of water and 5 cc. of 50 per cent sulfuric acid are added, and the alcohol is evaporated by boiling in a 200 cc. distilling flask under diminished pressure. The temperature is not allowed to go above 40°C. Crystals of the sulfate of the acetyl separate as the alcohol is removed. These are dissolved in about 15 cc. of alcohol which is filtered and added to a beaker containing 200 cc. of boiling water and 5 cc. of 50 per cent sulfuric acid. The addition of the first few drops of the alcohol solution of the acetyl does not cause a precipitate but further addition of the alcohol solution causes a precipitation in crystalline form of the free acetyl.

Purification of the acetyl may also be carried out by dissolving the acetyl sulfate in 25 cc. of alcohol and adding 5 gm. of sodium acetate and 10 cc. of 30 per cent sodium hydroxide. After the alcohol has been removed by boiling under diminished pressure, the disodium salt of the acetyl will separate in large, flat plates. The sodium salt may then be dissolved in alcohol and precipitated by addition to a boiling solution of dilute sulfuric acid as described above.

Many samples of the acetyl have been prepared and analyzed. Some of the results are as follows:

Analysis of the Acetyl of Thyroxin

Sample 1 5 26 mg. contained 3 20 mg of iodine = 60 91 per cent
"   2 5 20 "   "   3 17 " "   "   = 60 96 "   "
"   3 3 12 "   "   1 90 " "   "   = 60 85 "   "
"   4 5 12 "   "   3 11 " "   "   = 60 67 "   "
"   5 5 16 "   "   3 14 " "   "   = 60 83 "   "
"   6 5 10 "   "   3 11 " "   "   = 60 93 "   "
"   7 5 10 "   "   3 09 " "   "   = 60 61 "   "
"   8 5 04 "   "   3 05 " "   "   = 60 52 "   "
"   9 5 02 "   "   3 05 " "   "   = 60 74 "   "

97 9 mg of acetyl gave 90 1 mg of carbon dioxide and 16 2 mg of water; 5 10 mg contained 3 10 mg of iodine

	Carbon	Hydrogen	Iodine
	per cent	*per cent*	*per cent*
Calculated for $C_{13}H_{12}O_4NI_3$ ..	24 88	1 93	60 77
Found	25 09	1 85	60 86

The Sulfate of the Acetyl Derivative.—If the acetyl derivative is dissolved in alcohol and added to boiling dilute sulfuric acid, the acetyl precipitates in free form without the addition of sulfuric acid attached to the imino group. If, however, sulfuric acid is added to the alcohol solution of the acetyl and the alcohol is evaporated either at room temperature by a current of air or by boiling under diminished pressure at a low temperature, the sulfate of the acetyl separates in needle crystals.

Analysis of the Sulfate of the Acetyl

Sample 1 5 02 mg. contained 2 84 mg of iodine = 56 66 per cent
"   2 5 52 "   "   3 12 " "   "   = 56 52 "   "
"   3 6 2 "   "   3 51 " "   "   = 56 61 "   "

111 5 mg of the sulfate of the acetyl gave 94 8 mg of carbon dioxide and 19 7 mg of water, 5 52 mg. contained 3 12 mg of iodine

	Carbon	Hydrogen	Iodine
	per cent	per cent	per cent
Calculated for $C_{11}H_{13}O_6NI_3S$	23 07	1 92	56 36
Found	23 18	1 91	56 52

Although thyroxin is insoluble in pyridine, alcohol, ethyl acetate, or acetic acid, the acetyl is soluble in all these reagents. When the acetyl is precipitated from an alkaline solution by the addition of an acid, it is at first soluble in ether and may be extracted out of the water by placing in a separatory funnel with ether. After the acetyl has been prepared in pure form and dried, it is insoluble in ether.

The Di-Metal Derivatives of the Acetyl.[3]—The sodium, ammonium, and potassium salts of the acetyl are prepared as with thyroxin. 100 mg. of the acetyl are dissolved in 10 cc. of alcohol, to which are added 10 cc. of 30 per cent sodium hydroxide and 5 gm. of sodium acetate. The di-metal salt will separate in large, flat crystals if the alcohol is evaporated under diminished pressure. The temperature may be raised to that of boiling water without decomposition of the acetyl by the sodium hydroxide as no hydrolysis of the acetyl occurs under these conditions. If sodium chloride is substituted for sodium acetate in a solution similar to the one mentioned above and the alcohol is evaporated, the sodium salt of thyroxin separates in small plates.

The barium and calcium salts of the acetyl are prepared by dissolving 25 to 50 mg. of the acetyl in the least possible amount of alcohol, 2 to 3 cc., adding water and $\frac{1}{2}$ to 1 cc. of pyridine, the solution is boiled until the alcohol is volatilized, care being taken not to decompose the acetyl by prolonged boiling. To the pyridine solution of the acetyl a 40 per cent solution of barium or calcium chloride is slowly added, and the solution is heated to boiling. The alkaline earth salt of the acetyl will separate. It is readily soluble in an excess of pyridine. If the solution is heated for too long a time hydrolysis will occur. The salt may be filtered and washed without decomposition.

[3] In working with the acetyl, it is always necessary to dissolve the dry powder in alcohol before making it alkaline Unless this is done, some of the acetyl will spontaneously decompose during the solution of the powder in alkali

The reactions of thyroxin indicate that the molecule exists in both open- and closed-ring forms. The chemical properties of the acetyl are evidence that this derivative also exists in open- and closed-ring forms. The sodium salt of the acetyl washed with dilute acetic acid is completely hydrolyzed. When prepared in this way, however, its melting point is found to be 152°. When this material is dissolved in alcohol and added to boiling dilute sulfuric acid, the acetyl separates in closed-ring form, and the melting point is 238°. This suggests that in the sodium salt the acetyl exists in open-ring form, and hydrolysis of the sodium from the molecule leaves the open-ring structure. This is further corroborated by analysis of the silver salt.

Preparation of the Silver Salt of the Acetyl.—If the acetyl is dissolved in alcohol and then made alkaline with sodium hydroxide and the alcohol evaporated, the addition of ammonia and silver nitrate produces no precipitate. This is also true of thyroxin in the open-ring form. If 50 mg. of the acetyl are dissolved in 2 to 3 cc. of alcohol, to which are added 2 to 3 cc. of pyridine and 10 cc. of water, and the alcohol is removed by boiling, the addition of silver nitrate to the aqueous pyridine solution of thyroxin will cause a precipitate to form. This is not crystalline in nature, although under some conditions it may be possible to separate it in crystal form. The silver salt may be filtered on a Buchner funnel and washed with water without decomposition.

5 14 mg of the silver salt of the acetyl contained 2 26 mg of iodine = 44 06 per cent.
Calculated for $Ag_2C_{13}H_{12}O_5NI_3$ iodine = 44 35 per cent
"   " $Ag C_{13}H_{11}O_4NI_3$ "   = 51 91 "   "

The iodine content of the silver salt of the acetyl indicates the addition of 2 per cent too much silver for the disilver salt of the open-ring form of the acetyl, and 220 per cent too much silver for the mono-salt of the closed-ring form. These results, proving conclusively that not one but two atoms of silver had added to the acetyl, show that in an alkaline solution the acetyl exists in open-ring form.

The open-ring structure of the acetyl is also indicated by the increased solubility of the acetyl in weak bases such as pyridine, quinoline, and very dilute ammonia. The terminal carboxyl in

thyroxin is so weak that the acetyl derivative would not be soluble in these reagents in the closed-ring form.

Preparation of the Ureide.—Only pure thyroxin should be used in preparation of the ureide for the same reasons given under the preparation of the acetyl. 100 mg. of disodium or zinc salt of thyroxin are added to 10 to 15 cc of glacial acetic acid containing 200 mg. of potassium cyanate. Solution of the salt of thyroxin should be completed as the ureide is soluble in acetic acid. 5 cc. of water, 5 cc. of alcohol, and 5 cc. of 50 per cent sulfuric acid are added and the alcohol, water, and acetic acid are removed under diminished pressure, the same as in the preparation of the acetyl. The sulfate of the ureide is dissolved in 15 cc. of alcohol, filtered, and slowly added to a beaker containing 200 cc. of water and 5 cc. of 50 per cent sulfuric acid, which is heated to boiling. The ureide separates in crystal rosettes or needles

Sample 1. 4 78 mg of ureide contained 2 91 mg of iodine = 60.81 per cent.
" 2 5 04 "   "   "   "   3 07 "   "   "   = 61 03 "   "
" 3 5 02 "   "   "   "   3 05 "   "   "   = 60 74 "   "
Calculated for $C_{12}H_{11}O_3N_2I_3$ = 60 67 per cent

Preparation of the Methyl Ester.—100 mg. of the silver salt of thyroxin are suspended in 20 cc. of alcohol to which are added 4 to 5 cc. of methyl iodide. The crystals are occasionally stirred and allowed to stand at a temperature of 40 to 50° for several hours until decomposition of the silver salt is complete. This is indicated by separation of silver iodide in voluminous form. The silver iodide is removed by filtration. If the alcohol is allowed to evaporate, the dimethyl derivative crystallizes in the form of fine threads. They may be separated by formation of the sulfate similar to the preparation of the acetyl. 5 cc. of water and 5 cc of 50 per cent sulfuric acid are added to the alcohol filtrate from the silver iodide and the alcohol is removed by distillation under diminished pressure. The dimethyl derivative separates probably as the sulfate. It is insoluble in water, but soluble in alcohol. Treatment with alcoholic sodium hydroxide frees the carboxyl group, and the monomethyl derivative is then slightly soluble in aqueous sodium hydroxide. The methyl ester is very difficultly purified and has not been prepared containing a theoretical percentage of iodine. It appears to retain silver iodide even after repeated precipitation.

The Action of Nitrous Acid on Thyroxin.—25 mg. of thyroxin dissolved in 2 cc of dilute sodium hydroxide were added to the deamminizing chamber of the Van Slyke apparatus, and the usual procedure was followed, shaking for 3 minutes. 0 69 cc. of nitrogen was evolved. The apparatus was allowed to stand 9 minutes and was again shaken for 3 minutes; 0.06 cc. of nitrogen was obtained. 4 minutes later it was again shaken for 3 minutes and 0.04 cc. was obtained. The total, liberated in 22 minutes, showed 1.72 per cent amino nitrogen which is 72 per cent of the total nitrogen contained in the molecule.

Another 25 mg. sample of thyroxin, after 3 minutes shaking, liberated 0.75 cc. of nitrogen. This is equivalent to 1.60 per cent of amino nitrogen in the molecule and is 67 per cent of the total nitrogen.

25 mg. of the amino carboxyl salt form of thyroxin suspended in 2 cc. after 3 minutes shaking gave 0.08 cc. 10 minutes later, after 3 minutes more shaking, 0 06 cc., and 10 minutes later, after 3 minutes shaking, 0.04 cc.; total amount liberated = 0.18 cc. which is equivalent to 0.093 mg. of amino nitrogen, and is 16 per cent of the total nitrogen. 10 mg. of keto form of thyroxin suspended in 2 cc. of water gave no amino nitrogen.

The amino nitrogen in 25 mg. of the acetyl was determined as described above. After 3 minutes 0.11 cc., and after $10\frac{1}{2}$ minutes, 0.11 cc more of nitrogen were liberated. The nitrogen in the amino form amounted to 24 per cent of the total nitrogen in the molecule.

The nitrogen in isatin and indole, given off as amino nitrogen, was determined. 100 mg of isatin after 4 minutes gave 1.72 cc. and after 5 minutes more gave 0 41 cc of nitrogen This volume was equivalent to 1.13 mg. of nitrogen and amounted to 12 per cent of the total nitrogen present. 100 mg. of indole gave 1.27 cc. after 3 minutes, and 0.34 cc of nitrogen after 4 minutes more shaking. This is equivalent to 0.86 mg. and is 7 per cent of the total nitrogen in indole

Besides the action of nitrous acid on thyroxin liberating some of the nitrogen as amino nitrogen, a characteristic color reaction is produced. A few mg. of pure thyroxin, added to 5 cc. of alcohol containing three to four drops of 50 per cent hydrochloric to which are added five or six drops of 1 per cent sodium nitrite solution,

will develop a yellow color. This is increased by boiling. If the solution is cooled and concentrated ammonia is added until distinctly alkaline, a pink color is produced. This is a sensitive reaction for thyroxin, a distinct color being produced by one part of thyroxin in 40,000 parts of solution. If acetic or sulfuric acid is substituted for hydrochloric, the yellow color is not so deep and the addition of ammonia does not produce a pink color but gives a yellowish orange color. If the sample of thyroxin is impure, a yellow instead of a pink color is produced with ammonia. The acetyl and ureide derivatives give the same reaction, producing a pink color with ammonia.

The Pine-Splinter Reaction for Indole.—From 15 to 20 mg. of thyroxin are placed in a test-tube with 5 cc. of 30 per cent sodium hydroxide and sufficient water to carry the thyroxin into solution. As the excess of water is boiled off, thyroxin will precipitate as a disodium salt, but on further heating, after the water has been almost completely driven off and the temperature of the solution has been raised to between 100 and 200°, the disodium salt is again dissolved and a faint indole-like odor is given off. A pine-splinter moistened with hydrochloric acid is turned red by the vapors given off from the fusion.

The Effect of Thyroxin on Polarized Light.—1 gm. of thyroxin was dissolved in 20 cc. of alcohol and 7 cc. of water containing 300 mg. of sodium hydroxide. The solution was filtered and placed in a 2 dm. tube and its effect on polarized light was determined. No rotation of light could be determined. The solution of thyroxin was then placed in a 1 dm. tube. No rotation of the light could be determined.

The Effect of Hydrazine on Thyroxin.—If thyroxin is added to hydrazine hydrate, it immediately dissolves. If water is added, thyroxin remains in solution. The addition of carbon dioxide precipitates thyroxin as the amino carbonate. If the solution of thyroxin in the hydrazine hydrate is boiled after the addition of water, thyroxin precipitates as from ammoniacal solutions. No condensation with hydrazine, phenylhydrazine, semicarbazone, or hydroxylamine could be demonstrated when thyroxin was dissolved in alkaline alcohol to which these reagents were added, in acid alcohol to which these reagents were added, together with sodium carbonate or pyridine, or when dissolved in pyridine to

which the reagents were added.   Either decomposed products due
to heating at too high temperature or unchanged thyroxin were
recovered.

The Melting Points of Thyroxin and Its Derivatives.—The fol-
lowing melting points of thyroxin in its several forms and its
derivatives are recorded to illustrate the agreement between
different samples (Tables I to IX).   It is evident that among
some of the derivatives fairly large variations occur, but between
any two different forms the differences are much greater than
that found between the melting points of two samples of the
same derivatives.

The following salts of thryoxin and its derivatives were not
melted when heated to 260°: di- and monosodium salt of thyroxin;
di- and monopotassium salt of thyroxin; barium salt of thyroxin;
disilver salt of thyroxin; disodium salt of acetyl; disilver salt of
acetyl; calcium salt of acetyl.

TABLE I

The Melting Point of the Keto Form of Thyroxin.

No of sample	Slight browning	Sublime or mist	First droplets	Completely melted	Froth
125			248	250	250
32			241	248	248 9
123			235	247	247
124			240	246	246
81			243	245	246
91			241	242	243
107			241	242	242
115			239	242	242
119			239	241	241 5
117			237	241	241
97			236	240	241
28			195	240	241 5
80			239	240	240
59			238	239	240 5
51			236	239	240

TABLE II

The Melting Point of the Enol Form of Thyroxin.

No of sample	Slight browning	Sublime or mist	First droplets	Completely melted	Froth
40	190		200	202	205
41	180		200	205	205
42	190		200	204 5	204 5
43	160	190	200	204	204
44	160	190	202	208	208
46	170	190	200	202	204
49	165	190	203	206	206
50	165		196	201	201
53	150		198	203	204
55	160	190	200	202	204
108	160		198	204	205
109	180	198	206	208	208
110	170		200	202	203
111				203	205

TABLE III

The Melting Point of the Amino Hydrate Form.

No of sample	Slight browning	Sublime or mist	First droplets	Completely melted	Froth.
129			214	216	216 5
114			207	212	214

TABLE IV

The Melting Point of the Amino Carboxyl Salt Form.

No of sample	Slight browning	Sublime or mist	First droplets	Completely melted	Froth
86	215		219	220	221
88			216	218	221
89			216	218	219
95			219 5	221	222
100			222	223 5	223 5
105			222	223	223 5
112			216	220	221
113			217	221	222
118			221	222	222 5
120			221	224	224
121			222	224	225
127			218	222	223
130				225 5	226

TABLE V

The Melting Point of Acid Salts of the Amino Group.

	No of sample	Slight browning	Sublime or mist	First droplets	Completely melted	Froth
Amino sulfate	83		182	207	208	208 5
" "	93	162		202	204	204
" " . .	103	180		206	207	208
" oxalate . . .	104	180		206	207	208
" formate . .	101			202	205	208
" " . .	76	150		190	205	206.5
" "	84		180	202	204	204
" " . ..	82	160			204	205
" acetate. .	102			202	205	208
" carbonate	87	160		204	206	206

TABLE VI

The Melting Point of the Acetyl

No of sample	Slight browning	Sublime or mist	First droplets	Completely melted	Froth.
34			220	228	230
30			225	235	236
29			215	223	232

TABLE VII

The Melting Point of the Sulfate of the Acetyl.

No of sample	Slight browning	Sublime or mist	First droplets	Completely melted	Froth
27			111	150	200
25				122	190
24			100	106	150

TABLE VIII

The Melting Point of the Acetyl in Open-Ring Form.

No of sample	Slight browning	Sublime or mist	First droplets	Completely melted	Froth
8			150	160	174
35				155	155
11			146	152	216

TABLE IX

The Melting Point of the Ureide.

No. of sample	Slight browning	Sublime or mist	First droplets	Completely melted	Froth
21				225	225

SUMMARY.

The most important physical and chemical properties of thyroxin may be summarized as follows.

1. Thyroxin is a colorless, odorless, crystalline substance, insoluble in aqueous solutions of all acids including carbonic. It is soluble in sodium, ammonium, and potassium hydroxides, and is very slightly soluble in sodium and potassium carbonate. Besides forming salts with metals, thyroxin also forms salts with acids.

2. The iodine content of thyroxin and the iodine content of the sulfate salt were found to be 65 and 60 per cent respectively. This established the molecular weight of 585. Ultimate analysis and a study of the derivatives of thyroxin show the structural formula to be 4, 5, 6 tri-hydro-4, 5, 6 tri-iodo,-2 oxy,-beta indolepropionic acid.

3. In the presence of alkali metal hydroxides, thyroxin forms di-basic salts through the carboxyl and hydroxy groups. In the presence of carbonates, thyroxin forms mono-basic salts with the carboxyl group alone. The imino group forms salts with mineral and formic acids but not with acetic. The salts of mineral acids are soluble in alcohol, but no acid salt of thyroxin is appreciably soluble in water. Thyroxin forms derivatives through the imino nitrogen, such as the acetyl and ureide, and through its carboxyl and hydroxy groups, such as the dimethyl derivative.

4. Thyroxin exists in four distinct forms· (1) The keto form with the imino carbonyl groups, melting point 250°; (2) the enol form in which the hydrogen migrates from the imino to the carbonyl forming the hydroxy group, melting point 204°; (3) an open-ring form in which the elements of water enter the molecule between the imino and carbonyl groups forming an open-ring structure with amino and carboxyl groups, which exist in salt formation, called the amino carboxyl salt form, melting point 225°, and (4) a tautomeric form of this in which the elements of water

add to the nitrogen making the amino hydrate form, melting point 216°. If an acid is added to an enol form of thyroxin, the ring opens and the acid forms an amino-acid salt. The reason why weak organic acids including carbonic can add to the nitrogen of thyroxin-forming amino salts is because the ring is unstable in neutral aqueous solutions and the nitrogen tends to exist in the pentad state adding either the elements of water and forming an amino hydrate, or adding a carboxyl and forming an amino salt. These reactions could occur only with a strongly basic group. The amino group of analine and the imino group of indole or isatin are too feebly basic to react the same as thyroxin with weak organic acids.

5. Thyroxin is not easily oxidized or reduced, but will yield to both oxidation and reduction if sufficiently strong agents are used.

6. In alkaline solutions the iodine is broken off from the thyroxin molecule not as free iodine but as hypoiodous acid. This reaction is accelerated by sunlight. Sunlight also produces pink color compounds from the colorless thyroxin molecule.

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CARBONIC ACID AND CARBONATES IN COW'S MILK.

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The amount of carbonic acid present in cow's milk has been the subject of several investigations [1-5] The results reported by these investigators vary from 1 84 to 7.65 per cent of CO_2 by volume. It is not necessary to review the methods which have been employed to obtain the CO_2 from milk for measurement farther than to say that they have varied from extraction by means of a vacuum pump to expulsion by heat and have been more or less open to inaccuracy; nor is it important to consider previous methods used in measuring the amount of CO_2.

The results of our work are presented under the following divisions· (I) determination of CO_2 in milk, (II) relation of pasteurized milk to CO_2; (III) the form in which CO_2 exists in milk; (IV) the tension of CO_2 in milk.

I. Determination of CO_2 in Milk.

In measuring the amount of CO_2 in milk, we have used Van Slyke's method[6,7] in the determination of CO_2 in blood plasma, with certain modifications required to adapt the method to conditions present in milk.

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[2] Setschenow, *Z ration. Med* , 1861, x, 285

[3] Pfluger, E , *Arch ges Physiol* , 1869, ii, 166

[4] Thorner, W , *Chem Z* , 1894, xviii, 1845

[5] Marshall, C E , *Michigan Agric Exp Station, Special Bull No 16* , 1902

[6] Van Slyke, D D , *J Biol Chem* , 1917, xxx, 347

[7] Van Slyke, D D , and Cullen, G E , *J Biol Chem* , 1917, xxx, 291

Our method of procedure will be described under the five heads following: (1) drawing milk from cow's udder; (2) sampling the milk drawn; (3) determination of CO_2; (4) results obtained with different milks, (5) determination of CO_2 in milk by exhaustion.

1. Method of Drawing Milk.—In order to prevent loss of CO_2, it is of primary importance to have under complete control the method of drawing from the cow's udder the sample of milk in which CO_2 is to be measured. We have found the following method satisfactory: A silver milking-tube is inserted into the teat and the milk flows readily from the udder through this tube. To prevent the milk from coming into contact with the air, it is collected in a 100 cc. cylinder provided with a close fitting two-hole rubber stopper. Through one hole passes a glass tube extending to the bottom of the cylinder, and this tube is connected at its upper end by rubber tubing with the milking-tube. Passing through the second hole of the rubber stopper is a short glass tube which serves as an outlet for the air in the cylinder while it is being filled with milk. With this arrangement, the milk flows slowly and quietly into the cylinder without exposure to the air. The cylinder fills from the bottom upward, displacing the air until the milk fills the cylinder completely or even to slight overflowing. If desired, the surface of the milk can be protected from the air by a layer of thin paraffin oil but we have not found this necessary.

2. Taking Sample for CO_2 Determination —It is essential that the samples for the determination of CO_2 be taken from the cylinder as soon as practicable after the milk is drawn from the udder, because, on standing, the rising of the fat-globules to the surface changes the composition of the different layers of milk, and also there may be diffusion of the CO_2 from the upper surface if the top of the cylinder is not entirely filled with milk The best method of taking the sample from the cylinder is the following: One end of a 2 cc. pipette is connected to the tube leading to the bottom of the cylinder. Then one blows into the short tube, forcing the portion of milk last drawn from the udder to flow into the pipette until the milk overflows from the pipette, displacing all the air. The sample then passes from the lower part of the cylinder into the pipette without exposure to the air, without loss of CO_2, without being subject to appreciable change

of pressure, and without disturbance of the body of the milk in the cylinder. It has been found that the portion of milk first drawn from the udder and occupying the upper portion of the cylinder does not fairly represent the CO_2 content of the entire milking

3. Determination of CO_2.—The 2 cc. sample of milk is run into the Van Slyke CO_2 apparatus. Only slight modifications are required in the method as described by Van Slyke   We find that 5 per cent sulfuric acid and other mineral acids coagulate the milk and the coagulum interferes with the operation by clogging the apparatus, making it difficult to remove the CO_2 completely from the mixture.   Concentration of such acids greater than 5 per cent was also found unsatisfactory.   As the result of numerous experiments with different acids and concentrations, we find that a 20 per cent solution of lactic acid obviates all difficulties and meets all requirements, dissolving the casein quickly and completely and giving the same corrections as apply to the use of 5 per cent sulfuric acid   In all our work here reported, the amount of CO_2 was determined by absorption with a 5 per cent solution of NaOH instead of making a correction for inert gases. The results are the same by either process.

4. Results Obtained with Different Milks —In Table I we give the results furnished by the examination of twenty-five samples of cow's milk, drawn from the individual quarters of the udder. In addition to the amount of CO_2 in the milk, we give in each case the pH value and also the titration value expressed as cc. of alkali required to neutralize 100 cc of milk to phenolphthalein. The results are arranged in the order of the pH values

A study of the data embodied in Table I leads to the following statements

1. With the increase of the value of pH, that is, with decrease of hydrogen ion concentration, there is a general tendency for the CO_2 content of the milks to increase and for the degree of acidity, as measured by titration, to decrease.   This is not so marked between the pH values of 6.50 and 6.65 as it is above pH 6.65   Below pH 6 65, the CO_2 content varies between 7 and 10 per cent, while above pH 6 65 it increases somewhat uniformly from 12 at pH 6 70 to 86 at pH 7.16.

2. The acidity, as measured by titration, varies below pH 6.65 more or less irregularly between 16 and 20 cc. of 0.1 N alkali per 100 cc of milk, while above pH 6.65, the values decrease quite uniformly from 16 down to 4 cc.

3. In comparison with the values obtained by other workers, our lowest CO_2 values are about equal to, or higher than, the highest values previously reported. Thus, the highest figure heretofore published is 7.65 per cent of CO_2 by volume, while most of our values range from 8 up to 56 per cent in milks which

TABLE I

Amount of CO_2 in Cow's Milk

CO_2 (corrected) by volume	pH value	0 1 N alkali required to neutralize 100 cc of milk	CO_2 (corrected) by volume	pH value	0 1 N alkali required to neutralize 100 cc of milk
per cent		*cc*	*per cent*		*cc*
8	6 50	19 1	9	6 62	17 2
7	6 52	20 0	10	6 63	18 0
8	6 53	18 0	10	6 65	16 6
10	6 54	16 0	12	6 70	15 4
10	6 55	17 2	12	6 80	16 0
8	6 55	18 4	18	6 82	13 0
11	6 57	17 2	14	6 86	14 0
10	6 58	17 8	22	6 90	12 0
10	6 58	16 9	33	6 92	12 0
10	6 58	18 4	24	7 00	10 0
9	6 60	18 2	56	7 05	6 0
10	6 61	16 8	86	7 16	4 0
10	6 62	17.8			

appeared by ordinary inspection to be normal. In the milks examined by us which were known to be normal, the value most frequently found is about 10 per cent. However, our work has not been sufficiently extensive as yet to enable us to indicate positively a general average figure or an average range for normal milk.

5. *Determination of CO_2 in Milk by Exhaustion.*—It was desirable to ascertain whether it is possible to remove the CO_2 completely from milk for determination simply by vacuum exhaustion. We have found that this can be done by observing certain precautions The milk must be spread out in a thin layer during the

exhaustion. We placed 10 cc. of milk of the usual reaction (pH 6.5 to 6 65) in a 200 cc. separatory funnel and exhausted this for 2 minutes, turning the funnel end over end slowly in order to spread the milk in a thin layer over the interior surface of the funnel as completely as possible. Air was then admitted and the exhaustion repeated, after which the determination of CO_2 in the sample was made.[8]

Another portion (100 cc.) of the same milk was then placed in a 200 cc. separatory funnel and inverted. This was exhausted for 1 hour without any agitation of the milk, after which the amount of CO_2 in the sample was determined. We give the results of the two experiments in Table II.

Results of Removal of CO_2 from Milk by Vacuum Exhaustion.

Original milk		After exhaustion without agitation		After exhaustion with agitation in a thin layer.	
pH value	CO_2 by volume	pH value	CO_2 by volume	pH value	CO_2 by volume.
	per cent		*per cent*		*per cent*
6 54	10	6 57	4	6 60	0 0
6 86	14	6 92	7	6 98	0 0
6 92	22	7 00	9	7 06	0 0

These results make prominent certain points, as follows.

1. The CO_2 of milk can be completely removed by vacuum exhaustion, as shown by the results given in the last column of Table II, provided the milk is agitated and kept in a thin layer, the amount of milk used being small enough to permit control of these conditions. If the milk subjected to exhaustion is not agitated and exposed in a thin layer, the CO_2 is not completely removed, as shown by the results given in the fourth column of Table II In previous work done by others, in which vacuum exhaustion was relied upon to remove the CO_2 from milk, the removal was incomplete, owing to failure to observe the conditions required for complete exhaustion, as in the case of the results reported by Setschenow[2] and by Marshall.[5]

[8] Cullen, G E , *J. Biol Chem* , 1917, xxx, 369

2. The removal of CO_2 from milk results in an increase in the value of pH, that is, a decrease in the hydrogen ion concentration or, stated in another way, the milk becomes less acid, though to an amount that cannot be made appreciable by titration in normal milks In the first sample in Table II, the original milk, containing 10 per cent by volume of CO_2 has a pH value of 6.54, which increases to 6.57 when the CO_2 is reduced to 4 per cent, and which increases farther to pH 6.60 when the CO_2 is completely removed.

II. Relation of Pasteurized Milk to CO_2.

The observation stated in the preceding paragraph led us to make a study of some results which we had obtained in another investigation relating to the effect of pasteurization upon the reaction of milk.   We had noticed that pasteurization, if properly performed, is without observable effect in changing the hydrogen ion concentration of milk.   Our experiment was repeated with the modification that the CO_2 was completely removed from the milk by exhaustion before heating.   The two experiments gave results as recorded in Table III

TABLE III

Effect of Removal of CO_2 on Reaction of Pasteurized Milk.

Before removal of CO_2				After removal of CO_2			
Before heating		After heating at 63°C for 15 min		Before heating		After heating at 63°C. for 15 min	
pH value	CO_2 by volume	pH value	CO_2 by volume	pH value	CO_2 by volume	pH value	CO_2 by volume
	per cent		*per cent*		*per cent*		*per cent*
6 54	10	6 54	2	6 60	0	6 56	0

In studying the results of these two experiments, we notice:

1. In the milk in which CO_2 is not removed before heating, the pH value remains the same before and after heating, even though the per cent of CO_2 is decreased by the heating from 10 to 2 per cent.

2. In the milk in which CO_2 is completely removed by exhaustion before heating, the pH value decreases from 6.60 to 6.56, that is, the hydrogen ion concentration increases appreciably.

3. We have not yet carried our work far enough to furnish an explanation of the fact noted, but the inference appears justified that some chemical change occurs in the milk during pasteurization which results in an increase of hydrogen ion concentration, when CO_2 is absent, but that in the presence of CO_2 any change in the hydrogen ion concentration is, in some way not yet known, masked or offset by the loss of CO_2 which escapes from the milk during heating.

4. The decrease of CO_2 in pasteurized milk suggests that the CO_2 content of milk might be made the basis of a method for distinguishing pasteurized from normal milk. We are doing additional work in order to determine the limits of effectiveness of such a method.

III. Form in which CO_2 Exists in Milk.

It has been generally assumed that CO_2 exists in milk as uncombined carbonic acid. From the fact that the reaction of milk is less acid than that given by a corresponding solution of CO_2 in water, it appears probable that the CO_2 in milk is present in part as carbonic acid and in part as bicarbonate. To determine the proportion of CO_2 existing in milk as carbonic acid and as bicarbonate, two methods are available; (1) by calculation based on the application of the law of mass action, and (2) by direct determination   The results obtained by either of these methods can be regarded as only approximate, owing to the high dilution of CO_2 in milk.

1. *Calculation Based on Application of the Law of Mass Action.*— In a solution containing H_2CO_3 and $RHCO_3$, there exist in accordance with the law of mass action definite quantitative relations between the hydrogen ion concentration of the solution and the relative amounts of H_2CO_3. These relations are expressed by the following equations

$$C_H = K \frac{H_2CO_3}{C\acute{O}_3} = K \frac{H_2CO_3}{\alpha RHCO_3},$$

in which α is the degree of dissociation of $RHCO_3$ into R^+ and $HC\acute{O}_3$, and K is the ionization constant of H_2CO_3. From the foregoing, we have $\frac{H_2CO_3}{RHCO_3} = \frac{\alpha C_H}{K}$. Therefore, to determine the ratio

between bicarbonate and carbonic acid, we need only to know the values of K, C_H, and α for $RHCO_3$ (as $NaHCO_3$). According to Michaelis and Rona,[9] K equals 4.4×10^{-7}, the C_H value of average milk is about 0.25×10^{-6}; the value of α is difficult to determine with more than an approximate degree of accuracy under the conditions present in milk, but by a method similar to that of Michaelis and Rona, we obtain a value which makes the ionization of the bicarbonate in milk about 80 per cent. Applying these values in the equation, $\dfrac{H_2CO_3}{RHCO_3} = \dfrac{\alpha C_H}{K}$ we have $\dfrac{0.80 \times 0.25 \times 10^{-6}}{4.4 \times 10^{-7}} = \dfrac{10}{22}$ This result means that the CO_2 exists in milk in approximately the relation of one part of H_2CO_3 for two parts of bicarbonate, or that one-third of the CO_2 exists as H_2CO_3 and two-thirds as bicarbonate.

2. Proportion of Bicarbonate Acid and Bicarbonate Determined by Experiment.—The second method of ascertaining the proportion of CO_2 in milk present as carbonic acid and bicarbonate is based on the isohydric principle. A solution of carbonate containing a molecular concentration equal to that of milk would, if adjusted to the same hydrogen ion concentration, have approximately the same relative proportions of carbonic acid and bicarbonate. Milk is approximately a 0.01 N solution of H_2CO_3. In carrying out the details of our experiment, we dilute 10 cc of a 0.1 N solution of Na_2CO_3 to 100 cc. with water free from CO_2. Then we add a solution of 0.1 N HCl until the reaction is the same as that commonly found in milk (C_H, 0.25×10^{-6}). This requires 6.6 cc. of the acid. Of the 6.6 cc. of the 0.1 N HCl thus required 5 cc. are used to change Na_2CO_3 into $NaHCO_3$, leaving 1.6 cc. of 0.1 N HCl to act upon the $NaHCO_3$ and form H_2CO_3. In changing the 100 cc. of 0.01 N Na_2CO_3 solution into $NaHCO_3$, the resulting 100 cc. of 0.01 N $NaHCO_3$ has only one-half the neutralizing power of the Na_2CO_3 solution. Therefore, 1.6 cc of 0.1 N HCl neutralizes 3.2 cc. of the 100 cc of 0.01 N $NaHCO_3$ solution, forming 32 cc. of 0.01 N H_2CO_3 and leaving 68 cc. of 0.01 N $NaHCO_3$. These results furnish the ratio, 32 H_2CO_3 68 $NaHCO_3$, or, approximately, the ratio of 1 . 2 ; that is, one-third

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[9] Michaelis, L , and Rona, P , *Biochem Z* , 1914, lxvii, 182

part of the CO_2 exists in the solution as H_2CO_3 and two-thirds as $NaHCO_3$, a result which is in close agreement with that obtained by application of the law of mass action.

In this connection it is interesting to note that Marshall[5] states that CO_2 is not completely removed from milk by his method of vacuum exhaustion, owing, as he seems to think, to a slow generation of CO_2 in the milk. This is readily explained by the presence of bicarbonate in milk, which gradually gives up its CO_2 under reduced pressure as a result of the reaction of bicarbonate with some of the salts contained in milk.

IV. The CO_2 Tension in Milk

The CO_2 tension in milk is about the same as in most fluids of the animal body. Using McClendon's chart[10] we find by extrapolation of values that the CO_2 tension at 20°C of a 0.01 N solution, pH 6.6, is approximately equal to 50 to 55 mm of mercury. It is a matter of interest to notice that the CO_2 tension of blood under the conditions is given by him as 47 mm. Comparing this with the value for milk, one would expect a lower value in blood, because the latter is exposed to air in the lungs and, therefore, subject to loss of CO_2 by removal.

SUMMARY.

1. Milk is drawn from the cow's udder into a 100 cc. cylinder so as to fill the cylinder from the bottom upward, thus avoiding mixture with air or loss of CO_2. For the determination of CO_2, 2 cc. of milk are forced from the cylinder into the Van Slyke CO_2 apparatus without loss of CO_2. A 20 per cent solution of lactic acid is used to free the CO_2 in carbonates

2. In the case of twenty-five samples of milk drawn from separate quarters of the udder, the CO_2 varies from 7 per cent by volume to 86 per cent, the pH value varies from 6 50 to 7.16, in a general way increasing with the CO_2 content, the degree of acidity, as measured by titration, tends to decrease with increase of CO_2 content. In comparison with the results of other workers, the results obtained by us are higher. The CO_2 content of normal milk appears to be about 10 per cent by volume.

[10] McClendon, J. F , J. Biol Chem , 1917, xxx, 274.

3. It is possible to remove CO_2 from milk completely by vacuum exhaustion provided the milk is spread in a thin layer and kept in motion

4. When milk is pasteurized, the CO_2 content is decreased, but the pH value remains unchanged. However, if the CO_2 is completely removed before pasteurization, then the pH value appears to decrease slightly after pasteurization

5. CO_2 exists in milk as H_2CO_3 and as bicarbonate, probably $NaHCO_3$, the ratio being about one part of H_2CO_3 and two parts of $NaHCO_3$.

6. The CO_2 tension in milk is calculated to be about equal to 50 to 55 mm. of mercury at 20°C. in case of a 0.01 N solution with a pH value of 6.60.

CONDITIONS CAUSING VARIATION IN THE REACTION OF FRESHLY-DRAWN MILK.

By LUCIUS L VAN SLYKE AND JOHN C BAKER

(From the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva.)

The most sensitive method of measuring the reaction of milk is by measurement of the hydrogen ion concentration. This method has been employed by several investigators[1-7] in connection with the study of milk and it has been utilized in the work here presented.

The results of previous investigations show cow's milk, when freshly drawn, to have a reaction, expressed in terms of pH values, varying from 6.39 to 6.81, the range in most cases being between 6 50 and 6.65.

It appeared to us desirable to ascertain the extent of variation in large numbers of milks obtained directly from cows and to learn further, if possible, the causes of such differences. The results of our work will be discussed under the following headings: (1) Extent of variation of reaction in cow's milk, (2) variation in different quarters of the udder, (3) relation of reaction of milk to composition, (4) effect of abnormal conditions in the udder.

Extent of Variation of Reaction in Cow's Milk.

In undertaking to establish in our own experience the extent to which the reaction of fresh milk varies, we measured the hydrogen ion concentration in over 300 samples obtained from two herds of

[1] van Dam, W., *Rev gén Lait*, 1908, vii, 121.

[2] Allemann, O., *Biochem Z.*, 1912, lxv, 346

[3] Taylor, H. B, *J Proc Roy Soc N. S Wales*, 1913, lxvii, pt 2, 174

[4] Davidsohn, H, *Z. Kinderheilk*, 1913, ix, 14

[5] Clark, W M., *J. Med Research*, 1914–15, xxxi, 431

[6] Milroy, T H, *Pharmacol J*, 1914, xciii, 350

[7] Foa, C, *Compt rend Soc biol*, 1904, lix, 51

cows; one was a herd of Jerseys and the other of Holstein-Friesians. The samples used in our work were drawn separately from the individual quarters of the udder and the reaction was determined in each. In obtaining the samples, the first few streams of milk were thrown away and then enough more was drawn for use in our experiments. The results do not, therefore, represent the complete milking of the entire udder but only the foremilk of each quarter of the udder. The use of milk from individual quarters of the udder gives a greater variation in results than would be found if we used only samples representing the mixed milk of a complete milking of the entire udder. The use of the foremilk is very convenient for our purpose and is justifiable, since in our experience the reaction rarely changes appreciably in normal milks in portions successively drawn during milking, provided there is no excessive disturbance of the udder

In Table I we give summarized results of our work with over 300 samples of milk, the reaction being stated in terms of pH values.

TABLE I

		pH 6 50 to 6 60	pH 6 60 to 6 68	pH 6 68 to 6 76	pH 6 76 to 6 84	pH 6 84 to 6 92	pH 6 92 to 7 00	pH 7 00 to 7 20	Total number of samples
Herd 1	Number of samples	78	35	23	7	6	2	2	153
	Per cent of total	51	22 9	15	4 6	3 9	1 3	1 3	—
Herd 2	Number of samples	61	32	27	15	9	6	5	155
	Per cent of total	39 4	20 6	17 4	9 7	5 8	3 9	3 2	—

The samples of milk from the two herds of cows show quite as wide a range of hydrogen ion concentration as we have found in our entire experience up to the present time, working with a great variety of milks, though it is probable that somewhat wider variations may occur. Our results indicate that the reaction of fresh normal milk, expressed in terms of pH values, lies between 6.50 and 6.75 or 6.80. In Herd 1, 136 samples, or nearly 90 per cent of all the samples, and in Herd 2, 120 samples, or over 77 per cent, are below pH 6.76, that is, an average of 83 per cent of all

the samples examined for these two herds.   These figures are in agreement with results previously published, except that we find a small proportion of milks which are less acid, the pH values reaching as high as 7 2   We usually find that milks of such abnormally low acidity are sufficiently normal in appearance to pass the ordinary methods of market inspection when mixed in the commercial supply with other milks that are normal.   The wide range of values obtained by us is doubtless due to the fact that the samples of milk used in our work represent individual quarters of the udder and not the complete mixed milk drawn from the entire udder at one milking.   The mixed milk of a herd shows still smaller variation than that from single cows.

Variation of the Reaction of Milk in Different Quarters of the Udder.

It is a matter of interest to show at this point to what extent milk drawn from different quarters of a cow's udder may vary in reaction.   In Table II we give results obtained with twenty cows. The hydrogen ion concentration in these samples was determined approximately by means of brom-cresol purple used as an indicator, as described on page 364.   The results are given in two forms. The range of pH values from 6.50 to values above 7 is divided into seven groups, indicated by number, and after each such group number there is given in parenthesis the corresponding range of specific values.

A study of the tabulated data suggests the following points of interest.

1. The reaction found to be the more common is that showing the higher acidity.   Out of the 80 samples of milk drawn from the quarters of the udders of the twenty cows used in the work, 39, or nearly 50 per cent, are in Group 1, showing the highest acid reaction (pH 6 50 to 6.60), 21, or over 26 per cent, are in Group 2 (pH 6.60 to 6.68); 14, or over 17 per cent, are in Group 3 (pH 6.68 to 6.76), these three groups contain 92.5 per cent of the total.   Group 4 (pH 6.76 to 6 84) contains three samples, while the least acid groups, 5, 6, and 7, contain only one sample each.

2. These results indicate that the hydrogen ion concentration of normal mixed milk, when fresh, is that representing the most

acid reaction found by us (pH 6.50 to 6.60), the variations being in the direction of decreased acidity.

3. Comparing the different quarters of the udder in individual cows, we find that there are only four (Nos 16, 19, 20, 14) in which the reaction is the same in all quarters, and in these cases the re-

TABLE II

Results Showing Variation in Reaction of Milk Drawn from Different Quarters of Udder.

Cow No	Quarter of udder							
	Right front		Left front		Right hind		Left hind	
	Group	pH	Group	pH	Group	pH	Group	pH
16	1	(6 50–6 60)	1	(6 50–6 60)	1	(6 50–6 60)	1	(6 50–6 60)
19	1	" "	1	" "	1	" "	1	" "
20	1	" "	1	" "	1	" "	1	" "
14	2	(6 60–6 68)	2	(6 60–6 68)	2	(6 60–6 68)	2	(6 60–6 68)
2	2	" "	1	(6 50–6 60)	1	(6 50–6 60)	1	(6 50–6 60)
13	1	(6 50–6 60)	2	(6 60–6 68)	1	" "	1	" "
15	1	" "	3	(6 68–6 76)	1	" "	1	" "
10	3	(6 68–6 76)	2	(6 60–6 68)	2	(6 60–6 68)	2	(6 60–6 68)
12	5	(6 84–6 92)	3	(6 68–6 76)	3	(6 68–6 76)	3	(6 68–6 76)
1	2	(6 60–6 68)	2	(6 60–6 68)	1	(6 50–6 60)	1	(6 50–6 60)
5	2	" "	1	(6 50–6 60)	1	" "	2	(6 60–6 68)
9	3	(6 68–6 76)	3	(6 68–6 76)	1	" "	1	(6 50–6 60)
4	1	(6 50–6 60)	1	(6 50–6 60)	2	(6 60–6 68)	3	(6 68–6 76)
8	2	(6 60–6 68)	2	(6 60–6 68)	1	(6 50–6 60)	3	(6 68–6 76)
6	2	" "	3	(6 68–6 76)	1	" "	2	(6 60–6 68)
17	3	(6 68–6 76)	2	(6 60–6 68)	3	(6 68–6 76)	1	(6 50–6 60)
11	4	(6 76–6 84)	1	(6 50–6 60)	3	(6 68–6 76)	1	" "
18	1	(6 50–6 60)	1	" "	4	(6 76–6 84)	3	(6 68–6 76)
7	2	(6 60–6 68)	1	" "	1	(6 50–6 60)	6	(6 92–7 00)
3	1	(6 50–6 60)	2	(6 60–6 68)	7	(7 00–7 20)	4	(6 76–6 84)

action is that of the most acid groups, 1 and 2　In another examination of the same twenty cows, ten individuals were found to give a uniform reaction in the milk from all quarters, eight showing pH 6.50 to 6 60 and two, pH 6.60 to 6.68　In Table II the milk of only five animals (Nos. 12, 11, 18, 7, 3) departs markedly from the normal reaction, and in these cases the ab-

normal condition appears in only six of the twenty individual quarters.

4. While it is not the purpose of this article to discuss in detail the causes of these observed variations in reaction in the milk from the different quarters of the udder, we may state in passing that such variations must be due to some physiological condition of the animal, either a specific bacterial infection of the udder or a more general constitutional condition, such as variation in base and acid relations in the blood stream.

Relation of the Reaction of Milk to Composition.

Attention has been called to the fact that the reaction most commonly prevalent in freshly-drawn milk is the one that is most acid, and that the variation is all in the direction of decreased acidity, and, further, that the number of samples in which the acidity decreases is found to fall off rapidly with the greater decrease of acidity.

It was desired to ascertain, if possible, some of the conditions under which decrease of acidity occurs. Our attention was first turned to a study of possible relations that might exist between changes in reaction and changes in composition of milk. We made analyses of several samples of freshly-drawn milk in the case of cows which had previously been found to give milk varying noticeably in reaction from normal. Each sample of milk was drawn from one quarter of the udder, the entire contents being drawn, except in certain cases to which attention is called. All the samples were from cows whose milk was going into the local market supply. There was nothing abnormal that was observable in the appearance of the milk or of the cows, except that the milk with the least acid reaction had the characteristic bluish appearance of what we commonly call "thin" or "poor" milk.

The results are given in Table III, the analyses being arranged in the order of pH values, beginning with the most acid.

An examination of the data in Table III suggests that there are certain points of correspondence between the reaction of milk and the composition

We observe that, with a decrease of acidity, there is a marked tendency toward a decrease in the specific gravity and in the per-

centage of total solids, fat, solids-not-fat, casein, and sugar; but, on the other hand, an increase in albumin and proteins other than casein, and in the ash and also in the chlorine. There is, further, as we have shown in another article (p 338) an increase in CO_2 content with decrease of acidity

TABLE III

Results Showing Reaction of Milk in Relation to Composition.

Cow No	pH value	Total solids	Fat	Total proteins	Casein	Proteins other than casein	Sugar	Ash	Chlorine	Specific gravity
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
1	6 53	12 81	4 50	3 21	2 55	0 66	4 60	0 70	0 09	1 029
2	6 56	12 07	3 60	3 32	2 56	0 76	4 60	0 65	0 12	1 031
3	6 58	14 57	5 40	3 60	2 75	0 85	5 10	0 72	0 10	1 032
4*	6 60	13 30	3 80	3 31	2 62	0 69	5 20	0 69	0 11	1 030
5	6 70	12.10	3 60	3 21	2 50	0 71	4 40	0 86	0 13	1 030
6†	6 80	13 29	4 30	4 49	2 84	1 65	3 70	0 71	0 14	1 028
7	6 85	13 74	5 40	3 00	2 20	0 80	4 50	0 81	0 14	1 030
8	6.91	9 41	1 20	3 02	2 05	0 97	4 16	0 79	0 14	1 030
9	6.95	10 58	2 40	3 14	2 05	1 09	3 80	0 83	0 16	1 030
10	6 96	10.48	3 40	2 45	1 69	0 76	3 80	0 83	0 14	1 026
11	6 98	10 41	2 80	2 80	1 79	1 01	4 00	0 81	0 15	1 027
12‡	7 00	12 42	5 65	2 73	1 63	1 10	3 20	0 84	0 18	1 022
13	7 04	10 10	2 20	3 23	2 26	0 97	3 70	0 84	0 16	1 030
14*	7.06	8.85	2 80	2 64	1 50	1 14	2 60	0 91	0 22	1 021
15	7 15	9 13	2 10	3 14	1 71	1 43	3.00	0 89	0 21	1 026

*The sample was the last portion of milk drawn from the udder (strippings).

† The sample was from a cow in the last stage of lactation, being nearly "dry"

‡ The sample was from a cow just beginning the period of lactation, or "fresh in milk"

These findings raise the question as to whether there is any reason for the correspondence existing between the observed changes in reaction and composition. These changes are such as would be expected, if we were to add blood-serum or lymph to normal milk and they are also in agreement with the results re-

ported by others[8-16] who have worked with milk from diseased udders, though our samples were from udders which were apparently in normal condition.

This phase of the question brings us to a consideration of abnormal conditions of the udder in relation to the reaction of milk.

The Reaction of Milk in Relation to the Presence of Leucocytes and Bacteria in the Udder.

All the samples of milks used by us were from udders which were apparently in a condition of normal health under casual observation; but a special examination of those samples (Table III) showing a reaction indicated by pH values above 6.70 was made for leucocytes and streptococci. For the work done in making these examinations, we are under obligation to Miss Mildred C. Davis, City Bacteriologist of Geneva. Use was made of Breed's[17] method of direct-counting in the milk. The results of the work are given in Table IV.

The results in Table IV indicate in a general way that decreased acidity in fresh milk is related to infection of the udder. Decrease of acidity is shown to be associated with increase of leucocytes, provided acid-producing streptococci are not present in sufficient numbers to neutralize such effect. Thus, in No. 7, we have a milk not far from normal in reaction, even though it contains a large number of leucocytes (20 million per cc.), a number which in Samples 14 and 15 gives a marked relative decrease in acid reaction; but this condition in No. 7 appears to be accounted for by the relatively large number of acid-producing streptococci (1 million per cc.), the acid produced offsetting the decrease of acidity caused by leucocytes.

[8] Storch, V , Jahresb. Thierchem., 1884, xiv, 170; 1889, xix, 157

[9] Hoyberg, H M , Z Fleisch -u. Milchhyg , 1911, xxi, 1

[10] Fetzer, L W , Eighth Internat. Cong Applied Chem , 1912, xix, 111

[11] Chrétien, M , Hyg viande et lait, 1912, vi, 382

[12] Allemann, O , Milkwirtsch. Centr , 1915, xliv, 122

[13] Zaribnicky, F., Arch. wissensch u prakt tierheilk , 1913–14, xl, 355

[14] Henderson, J B , and Meston, L. A , Chem. News, 1914, cx, 275, 283, 1915, cxi, 51.

[15] Bahr, L , Z Fleisch -u Milchhyg., 1913-14, xxiv, 251, 288, 370, 398, 472.

[16] Foa, C , Compt. rend Soc biol , 1905, lix, 51

[17] Breed, R S., New York Agric Exp Station, Techn Bull. 49, 1916

TABLE IV

Relation of Leucocytes and Streptococci to the Reaction of Milk

Cow No	pH value	No of leucocytes per cc	No of streptococci per cc
		millions	*millions*
6	6 80	1	0 1
7	6 85	20	1 0
8	6 91	3	Small number
9	6 95	5	" "
10	6 96	10	" "
11	6 98	8 4	" "
12	7 00	4	0 2
13	7 04	3	Small number
14	7 06	20	0 5
15	7 15	21	0 3

A further study was made of five samples of milk in which the approximate pH value was determined by means of brom-cresol purple used as an indicator, the microscopic examination for leucocytes and streptococci being made by Miss Davis. The results are given in Table V.

TABLE V

Results of Examination of Milk for Leucocytes and Streptococci

Sample No	Approximate pH value	Cells	Acid production
1	6 68–6 76	Many leucocytes Streptococci, innumerable and in clumps at bottom of tube	Marked acid production by clumps at bottom of tube within 1 hour after milking
2	6 72–6 80	5 million leucocytes per cc Streptococci, innumerable, separate, and in clumps	Marked acid production by clumps at bottom of tube within 30 minutes after milking
3	6 76–6 84	Many leucocytes Streptococci, innumerable, separate, and in clumps at bottom of tube	Marked acid production by clumps at bottom of tube within 30 minutes after milking
4	Over 7 00	Leucocytes, innumerable, few streptococci	No acid production apparent
5	" 7 00	Leucocytes, innumerable, few streptococci.	No acid production apparent

Summarizing the results embodied in Table V, we notice

1 In Samples 4 and 5, containing innumerable leucocytes and showing evidence of the presence of garget, there were few streptococci and the acidity was lowest. On standing a few hours, these samples furnished no evidence of increase of acidity.

2 In Samples 1, 2, and 3, which were the more acid ones, there were immense numbers of streptococci, especially in clumps, and while there were also many leucocytes, there was an increase of acidity within a very short time after the milk was drawn from the udder.

3. It appears highly probable that the greater acidity in the milks containing enormous numbers of streptococci is due to the formation of acid by these organisms, especially in view of the fact that milk from diseased udders, containing large numbers of leucocytes with few streptococci, shows the lowest degree of acidity found in fresh milk   In other words, we find milks that contain large numbers of leucocytes and are abnormally low in acidity when streptococci are present in only small numbers or entirely absent usually show appreciably higher acidity when streptococci are present in large numbers.

A Suggested Explanation of the Decreased Acidity in Abnormal Milks.

We have already stated that the correspondence existing between the observed changes in reaction and composition of milk may be accounted for on the supposition that the change in the direction of decreased acidity is due to the presence of blood-serum or lymph. Decrease of acidity in fresh milk is observed in case of diseased udders and may, therefore, be due to the direct filtration of blood-serum or lymph into the lumen of the alveoli without transformation by the gland cells or through lesions caused by bacterial activity. This view harmonizes with several facts. (1) It is in harmony with the changes in composition of the milk, (2) it is in agreement with the hydrogen ion concentration shown by normal milk (pH 6 50 to 6.60), and that shown by blood-serum (about pH 7 60), (3) it harmonizes with the variation found by us in the CO_2 content of milk, normal milk containing about 10 per cent by volume and blood-serum, 65 per cent, and (4) it is in agreement with the increasing number of leucocytes found in the less acid milks.

Further proof of the presence of blood-serum or lymph in most of the abnormal milks (pH 6 90 to 7.20) examined by us is the existence of fibrin in such samples, as shown by Doane's method.[18]

Another method of proof was undertaken, which was to ascertain if glucose is present in the abnormal milks under discussion If serum passes unchanged into milk, glucose should be present in such abnormal samples in appreciable amount. We were, however, unable in any case to find the slightest trace of glucose in these abnormal milks. If glucose is absent from the serum present in these milks, the glucose in the blood-serum must be changed into another compound by some agent present in the milk in the udder, which might be udder cells or some enzyme. The question calls for further investigation.

The method used by us for the detection of glucose is the following. Proteins are precipitated by 70 per cent alcohol and the filtrate evaporated to dryness. This residue is extracted first with ether and then with hot 95 per cent alcohol. The alcoholic extract is evaporated to dryness and the residue again extracted and the extract evaporated to dryness, after which the residue is extracted with a small amount of ether. This residue is used for the osazone test. Glucose added to milk can be recovered easily by this method.

<div align="center">SUMMARY.</div>

1. The object of the investigation was the study of the extent and causes of the variation of the hydrogen ion concentration in freshly-drawn cow's milk.

2. In the case of over 300 samples of fresh milk, the pH value varied from 6.50 to 7.20, being under 6.76 in 80 per cent of the samples

3. In the case of the milk from 20 cows, it was found that the pH value of the milk from different quarters of the udder varied greatly but in most cases the variations were not large.

4. The pH value is found to vary with the composition of the milk. In general, with a decrease of acidity, there is a marked tendency toward a decrease in specific gravity, and in percentage of fat, total solids, solids-not-fat, casein, and lactose, but an increase in proteins other than casein and in ash and chlorine.

[18] Doane, C. F., *Maryland Agric Exp. Station, Bull. 102.* 1905.

5. These changes in composition are such as would be expected in case blood-serum or lymph were added to normal fresh milk. Abnormal conditions in the udder might cause such addition.

6. Examination of milks of abnormally low acidity, having a pH value above 6.80, indicates that the reaction is accompanied by the presence of large numbers of leucocytes, though the reaction in such cases may be neutralized by the presence of large numbers of acid-producing streptococci.

7. While the belief in the presence of blood-serum or lymph in such milk is supported by several considerations, a careful test for glucose proved negative.

A METHOD FOR THE PRELIMINARY DETECTION OF ABNORMAL MILK BASED ON THE HYDROGEN ION CONCENTRATION.

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(From the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva)

INTRODUCTION

In the official inspection of market milk, the primary object is to detect samples that are abnormal in composition as well as in sanitary character   The full examination of a large number of samples for the purpose of detecting an occasional abnormal one involves a relatively large amount of inefficiency in attaining the object   Attempts have been made to minimize the labor of inspection by using some quick and simple method which would serve the purpose of enabling one to detect suspicious samples; and only those samples which showed some evidence of abnormality by such preliminary test would be selected for further detailed examination in the laboratory, in order to confirm or disprove the suspicion   In examining market milk for the purpose of quickly identifying abnormal samples, inspectors have been limited in their methods to the use of the hydrometer or lactometer, except that in some cases the senses of smell, taste, and sight could also be employed to advantage   The determination of the specific gravity of milk has found its chief use in enabling one to select samples which appear to give evidence of being watered or skimmed   It has been repeatedly shown that specific gravity as a basis for accurate judgment in identifying abnormal milks may be wholly misleading   The need of a more comprehensive and reliable method has long been realized

The Relation of Hydrogen Ion Concentration to Normal and Abnormal Milks.

In making a study of the hydrogen ion concentration of freshly-drawn milks, normal and abnormal, and also of normal milks subjected to various conditions of change, it was found that the hydrogen ion concentration is very sensitive to certain conditions, among which are (1) production of acid by bacteria, (2) the addition of formaldehyde solution, (3) the addition of acids, (4) heating above a certain temperature, (5) abnormal or diseased milks, (6) addition of water, (7) addition of alkali or alkaline salts, (8) removal of fat. The first four conditions increase the hydrogen ion concentration, that is, render the reaction of the milk more acid than normal, while the other conditions render the reaction of the milk less acid than normal.

It occurred to us that, if it were possible to obtain an indicator having a neutral point near that of normal milk and yet showing an appreciable color in normal milk, which would be sufficiently sensitive to show observable change of color with slight change of hydrogen ion concentration, such an indicator might find application as the basis of a method to be used for the purpose of quickly indicating the probability of normality or abnormality in a milk. The first suggestion of an indicator meeting these conditions came to us in connection with the work published by Clark and Lubs[1] on "A substitute for litmus for use in milk cultures." They made use of a dye, known as dibrom-ortho-cresol-sulfon-phthalein, the name being shortened for convenience to "brom-cresol purple." This dye was found by them to possess properties which make it a reliable and brilliant indicator for the colorimetric determination of hydrogen ion concentration in milk.[2]

We first made use of brom-cresol purple in testing its applicability to the detection of increased acidity in milk when formed by bacterial action, and found that it is extremely sensitive in comparison with phenolphthalein, which is the indicator in common use in titration for the determination of the degree of acidity

[1] Clark, W. M., and Lubs, H. A., *J Agric. Research*, 1917, x, 105.
[2] This dye can be purchased from Hynson, Wescott, and Dunning, Baltimore, Md. In ordering this dye the full name should be used.

in milk. Further extension of the use of brom-cresol purple demonstrated its practicability in detecting other conditions, especially those mentioned above.

<center>OUTLINE OF PROPOSED METHOD.</center>

Before describing the detailed operation of the method, we will give a brief statement, outlining its main features. The use of brom-cresol purple in this application to the preliminary detection of abnormal milks consists in adding to one drop of a saturated water solution of the dye 3 cc. of milk and then observing the color. In the case of average milks that are normal in character, such, for example, as good market milk, the color is very uniform, being a bluish-gray. In the case of a milk giving a color differing appreciably from this, there is ground for suspicion that it is not normal The color given by different milks may be lighter or darker, ranging from a bright yellow at one extreme to a deep blue at the other. *The color is made lighter by acids, acid salts, formaldehyde solution, and also by heating above the usual point of pasteurization The color becomes deeper blue in the case of milk from diseased udders, watered milk, skimmed milk, and milk containing added alkali or an alkaline salt.*

If a preliminary test with brom-cresol purple gives a color lighter than in the case of normal milk, then a sample can be taken by the inspector to be used in making a further detailed examination in the laboratory for acidity, formaldehyde, and over-heating If the color is darker than normal, then a sample is taken to ascertain whether the abnormality is due to the addition of water, alkaline salts, removal of fat, or to the presence of milk from a diseased udder.

Attention should be called here to some conditions which modify the characteristic color given by brom-cresol purple solution with average normal milk.

1. Effect of High Percentage of Milk-Fat —The presence of extra fat, as in the case of rich milks (5 per cent or more) gives an appreciably lighter color than in the case of the ordinary market milks containing 3 to 4 per cent of milk-fat.

2. Effect of High-Colored Milk —In the case of milks produced by cows at fresh pasture, the milk has a decided yellow color which modifies the color reaction with brom-cresol purple

3. Effect of the Removal of Milk-Fat —When fat is removed from milk, the resulting skim-milk gives with brom-cresol purple a darker color than does the same milk before the removal of fat.

These differences in color are due to the fact the fat-globules do not give the same reaction color as normal milk with brom-cresol purple and they thus modify by their presence the color of the indicator in the milk   The fat dissolves some of the dye which always appears yellow in the fat.   The effect of milk-fat upon the reaction color can be readily observed if one notices the color at once after mixing the milk and the solution of brom-cresol purple and then again after the fat-globules have risen to form a cream layer at the upper surface   It will be seen that the color is lighter at the start than it is after the cream has risen and, further, that the cream layer shows little or no color.   The difference is more marked with increase of milk-fat

OPERATION OF METHOD

1 Preparation of Indicator

Brom-cresol purple is ground to a fine powder and dissolved in distilled water to saturation, about 0.1 gm. being used for 100 cc of water   Saturation can be hastened by heating the mixture on a water bath, then cooling to room temperature, and filtering The saturated solution contains about 0 09 per cent of the dye

2 Apparatus.

The only apparatus required is the following · a burette, test-tubes, a pipette, and a test-tube holder

The burette is used for the purpose of measuring the indicator The delivery is so controlled that each drop measures 0 05 cc.

The test-tubes which we have found most convenient for use in making the test are flat-bottomed specimen tubes made of Pyrex glass, holding about 8 cc   They are about 4 inches long and $\frac{1}{2}$ inch in diameter.   It is essential that all the tubes used should be uniform in color and in thickness of walls

We have found it convenient to provide a special holder for these tubes, making it easy to compare the color by arranging the tubes in a line side by side in close contact without concealing any portion of the milk column.

Ordinary 3 cc pipettes are used for measuring the milk to be used.

3. Performing the Operation.

The different steps in carrying out the details of the method consist of (a) measuring the indicator, (b) measuring the milk, (c) observation of the color, and (d) interpretation of the results.

(a) *Measuring the Indicator* —The test-tubes are placed in the holder with the open end up. The burette is filled with the solution of brom-cresol purple, and the stop-cock so adjusted that it delivers drops measuring 0 05 cc at the rate of about one drop in 2 seconds The test-tubes are placed under the burette tip, one by one in turn, exactly one drop being allowed for each tube   The delivery is so controlled that each drop falls free from the burette tip into the test-tube without touching the walls of the tube before the drop separates from the burette tip   This procedure enables one to deliver the same amount of indicator into each test-tube with rapidity   There are two advantages in using only one drop of indicator.   The first is the minimum dilution of the added milk and the second is that the tubes can be carried about without danger of losing the indicator.

(b) *Measuring the Milk Sample* —The milk is added to each test-tube with a 3 cc pipette and is thoroughly mixed with the indicator, which may be conveniently done either by shaking the tube or by drawing the mixture of milk and indicator into the pipette and allowing it to flow back into the test-tube   In our experience the proportion of 3 cc of milk and one drop of brom-cresol purple solution enables one to observe the shades of color to best advantage in most cases, but in some cases we have obtained somewhat better results in observing color changes with 5 cc of milk for one drop of indicator   ·

(c) *Observation of Color Change* —The ability to distinguish shades of color in the change of reaction in milk is the chief point of difficulty and, therefore, the observation of shades of color

constitutes the main source of weakness in its application. The method cannot be used successfully by one whose eye is lacking in appreciation of different shades of color to such an extent that training does not enable one to overcome such deficiency. However, in our experience, any person with normal sensitiveness to color changes can acquire the ability to observe such changes as take place in milk treated with brom-cresol purple solution with an accuracy which will make the application of the method useful. The fundamental difficulty lies in the lack of a fixed color standard which is applicable under all conditions as a basis of comparison. In the examination of market milks, it usually suffices to assume that a large proportion of the samples is normal in reaction and that, therefore, in a collection of numerous samples, those which give the same color with the solution of brom-cresol purple are generally normal, while those samples which are lighter or darker are open to the suspicion of being abnormal in some respect and should be further examined by supplementary methods. It will be well usually, however, especially for those who are unaccustomed to the use of the method, to prepare a series of known standardized colors to be used as a basis of comparison in observing the reaction of unknown milks to which the test is applied

Preparation of the Color Standard —The prepared color standard represents approximately certain ranges of hydrogen ion concentration. Briefly stated, the preparation of such a standard consists in adding increasing amounts of standard alkali to a mixture of normal milk and brom-cresol purple solution. The preparation of the series of standard colors to be used for comparison is carried out in the following manner.

(1st) Selection of Milk.—The milk to be used in the preparation of the color standard should meet two requirements. First, it should have approximately the same general composition as that of the milks to be examined, and, second, it should have a normal reaction. In respect to composition, usually any normal market milk containing between 3 and 4 per cent of fat will be satisfactory in the inspection of market milks. When milks containing over 4.5 per cent of fat are to be tested, it is well to use for the color standard a milk containing about the same percentage of fat. In the case of partially skimmed milks, milk with less

than the normal percentage of fat should be used   The differences in color caused by the presence of varying percentages of fat can be largely overcome, when necessary, by removing the cream with a centrifuge from all the milks to be examined, using skim-milk in the preparation of the color standard.   However, it should be stated that the color given by skim-milk obtained from milk rich in fat may be slightly different from that given by skim-milk obtained from milk poor in fat   After one has had some experience in studying the effect of different milks upon the solution of brom-cresol purple, it will be found that the matter of the selection of the milk for making a color standard is simpler than it might appear from the foregoing statements.

In respect to the reaction, the milk to be used in preparing the color standard should not have an acidity in excess of an equivalent of 18 cc of 0 1 N NaOH per 100 cc. of milk   The determination is made by titrating 10 cc of milk without dilution with the alkali, using 0 5 cc of a neutralized, alcoholic solution of phenolphthalein for indicator · The reaction of the milk to be used for the color standard can be further tested by adding brom-cresol purple solution and comparing the resulting color with that given by this indicator with samples of milk of known normal character   If the reaction is uniform with that of the known normal milks, the milk can be satisfactorily used for the color standard   Usually a milk having an acidity equivalent to 1 8 cc of 0 1 N NaOH per 10 cc. of milk is found to be satisfactory

(2nd) *Preparation of Standard Color Series* —Measure eight portions of milk of 10 cc each into separate test-tubes and to each portion add the amount of 0 1 N NaOH indicated below.

Test-tube No	1	2	3	4	5	6	7	8
No of drops of 0 1 N NaOH	0	2	4	6	8	10	12	14

The alkali is added to the tubes from a burette, using the same precautions in regard to uniform size of drops and their delivery as have been already given for measuring the brom-cresol purple solution   The alkali and milk are thoroughly mixed.   Of the mixture in each tube take 3 cc and one drop of brom-cresol purple solution, making a series of eight mixtures contained in the kind of test-tubes previously described.   These give a range

of colors to be used as a standard for comparison in testing unknown milks. The reaction color in each tube corresponds approximately to the following pH values.

No in series.	1	2	3	4	5	6	7	8
cc of 0 1 N NaOH used	0	0 1	0 2	0 3	0 4	0 5	0 6	0 7
pH value	6 5 to 6 6	6 6 to 6 67	6 67 to 6 75	6 75 to 6 82	6 82 to 6 90	6 90 to 6 98	6 98 to 7 05	7 05 to 7 13
Symbol for reaction color	N	N−1	N−2	N−3	N−4	N−5	N−6	N−7

As a matter of convenience in tabulating results, we append a series of symbols to indicate the pH values, N standing for normal reaction and N followed by the minus sign and figures ranging from 1 to 7, indicating decreased acidity corresponding to increasing pH values

It should be emphasized here that this method gives the reaction only approximately and the accuracy of the results may be interfered with by various conditions apart from the true reaction   Of such interfering factors, one which is especially influential in affecting the color is the degree of opaqueness. Milks of relatively low acidity tend to be less opaque than those of normal acidity. As a result the surface color observed is reflected from a greater depth and with a corresponding increase in the intensity of the color in the presence of brom-cresol purple solution, thus indicating a reaction less acid than that indicated by the standard. When necessary, this source of interference can be obviated by so diluting the standard series with water after the addition of alkali and before the addition of the brom-cresol purple solution that the milk to be used as a standard will resemble in its appearance of opaqueness that of the unknown milk. The milk thus diluted is then used in making up a special standard series by addition of indicator   While the addition of water changes the reaction of the standard somewhat, the test of the reaction by the color is made more accurate than if the standard is undiluted.

Another factor which may render less accurate the use of the standard in determining the reaction of unknown milks is the initial reaction of the milk used for the standard   If, for example, the initial reaction is pH 6 5 in one case and pH 6 6 in another,

there would be a difference of 0 1 pH in applying the standard to the reaction of unknown milks.

(d) *Interpretation of Color.*—In examining the samples of milk in the test-tubes after addition of the milk to the brom-cresol purple solution, one makes comparison with the prepared color standard; or, in the absence of such a standard, one selects those samples which differ in color markedly from those samples which, according to experienced observation, appear normal, either before or after the separation of the cream Such samples as appear to be abnormal by showing a deeper blue shade of color, indicating decreased acidity, are open to the suspicion of being watered, or skimmed, or treated with alkaline salts, or containing excessive numbers of leucocytes as in milk from diseased udders. Which of these suspicions is justified can be ascertained by the determination (1) of the freezing-point, (2) of the percentage of milk-fat or the ratio of fat to proteins, (3) of the specific gravity, (4) of the total solids, (5) of the presence of alkaline salts, especially sodium bicarbonate and borax, (6) of the numbers of leucocytes by direct microscopic examination by Breed's method,[3] and (7) of CO_2[4] by Van Slyke's method modified by us for use in connection with milk

In the case of samples showing a color lighter than normal with the brom-cresol purple solution, indicating an abnormal degree of acidity, there is awakened the suspicion of bacterial acid production, the presence of formaldehyde, overheating, or the presence of added acid salts, or the lighter color may be due to a high percentage of milk-fat Which of these indications is correct is determined as follows. A direct count of the number of bacteria[3] is often sufficient   If this fails to show the presence of excessive numbers of bacteria, then a test should be made for the presence of formaldehyde, and, if this is not present, the percentage of milk-fat is determined, and, further, in order to see if the light color is due to overheating, the determination of carbon dioxide should be made and Storch's test may also be applied

[3] Breed, R S , *New York Agric Exp Station, Techn Bull 49*, 1916
[4] Van Slyke, L L , and Baker, J C , *J. Biol Chem* , 1919, xl, 337

Relation between the Reaction of Milk and the Presence of Added Water and of Garget.

We have made application of this preliminary test extensively in the case of market milk and especially in connection with watered milk and milks containing garget and also milks with a high bacterial content

Of 570 samples of market milk to which the test was applied, 64 samples, of which 52 showed decreased acidity, were selected by the brom-cresol purple test for more detailed examination in relation to the presence of added water and high leucocyte content   The results of the work are summarized in Table I   Each sample of market milk represented a single can of milk as delivered at the milk station by the producer.   Milk from the same producer was sampled on several different days in those cases in which there was cause for suspecting the milk to be abnormal. Most of the samples giving a normal color reaction (N) were selected from the milk of producers whose milk appeared on previous inspection to show signs of being watered but which gave a normal reaction at the time of this sampling.   There are given in addition results in the case of a few samples (Nos. 23 to 28) of milk obtained from different quarters of the udders of cows, some of which were known to have udder infection, and there are also three samples (No 6) of normal mixed milk from our Station herd

An examination of the results contained in Table I leads to the following summarized statements: •

1. Of the selected 64 samples of market milk, 52 show a color reaction less acid than normal and 12, a normal reaction

2 In all the samples giving a normal color reaction, the depression of the freezing-point varies between $- 0.54$ and $- 0.57°$. In the 52 samples of market milk showing a reaction less acid than normal, constituting nearly 10 per cent of all the samples examined by the color test, 39 samples show a depression of the freezing-point varying from $- 0.468$ to $- 0.538°$, thus indicating the presence of added water   In 18 samples giving N-1 color reaction (pH 6.6 to 6 67), 10 samples show watering by the

TABLE I

Results of Examination of Market Milk by Brom-cresol Purple Test.

Herd No	Reaction	Depression of freezing-point	Garget	Herd No	Reaction	Depression of freezing-point	Garget
		degree				*degree*	
1	N	0 569	0	13	N-2	0 538	0
2	"	0 558	0	13-a	"	0 509	0
3	"	0 558	0	14	"	0 477	0
4	"	0 560	0	14-a	N	0 548	0
4-a	"	0 560	0	14-b	N-2	0 530	0
6	"	0 548	0	14-c	"	0 512	0
5	"	0 552	0 ·	14-d	"	0 490	0
6-a	"	0 555	0	14-e	"	0 523	0
6-b	"	0 562	0	14-f	"	0 503	0
7	N-1	0 536	Present	14-g	"	0 513	0
7-a	N	0 546	0	14-h	"	0 520	0
7-b	N-1	0 546	Present	14-i	"	0 525	0
7-c	"	0 550	"	14-j	"	0 500	0
7-d	"	0 536	"	14-k	"	0 530	0
7-e ·	N	0 558	0	15	"	0 518	0
7-f	N-1	0 552	Present	15-a	"	0 536	Present.
7-g	"	0 549	"	15-b	"	0 543	"
7-h	"	0 549	0	15-c	N-1	0 518	0
7-i	N	0 553	0	16	N-2	0 523	0
8	N-1	0 528	0	16-a	"	0 506	0
8-a	"	0 547	0	16-b	N-1	0 512	0
8-b	N-2	0 530	0	16-c	N	0 556	0
8-c	N-1	0 545	0	16-d	N-2	0 496	0
8-d	"	0 538	0	17	"	0 494	0
8-e	N-2	0 541	0	18	N-3	0 519	0
8-f	N-1	0 563	Present	19	N-2	0 530	0
8-g	N-2	0 540	0	20	N-1	0 491	0
9	"	0 518	0	21	"	0 531	0
9-a	"	0 531	0	22	N-2	0 540	0
9-b	"	0 512	0	22-a	"	0 540	0
9-c	N-1	0 530	0				
10	N-2	0 500	0	23	N	0 558	0
10-a	N-1	0 512	0	24	"	0 558	0
10-b	N	0 558	0	25	N-4	0 540	Present.
10-c	N-3	0 470	0	26	"	0 563	"
11	"	0 468	0	27	N-5	0 558	'
12	"	0 519	0	28	"	0 548	"

freezing-point determination, while 8 do not. Of these 8 samples, the decreased acidity is due to the presence of garget in 5 cases. The other 3 cases are on the border line as shown by the freezing-point depression. In 30 samples showing N-2 color reaction (pH 6.67 to 6.75), 25 contain added water according to the freezing-point, while 5 are just on the border line, showing a freezing-point depression varying from $-0.54°$ (in 3 cases) to $-0.543°$. In the case of 4 samples (10-c, 11, 12, 18), giving a color reaction of N-3 (pH 6.75 to 6.82), all showed the pres-

TABLE II.

Results of Examination by Herds.

Herd No	No. of times examined	No. of samples with subnormal reaction	No. of samples watered	No. of samples with garget
7	10	7	0	6
8	8	8	3*	1
9	4	4	4	0
10	4	3	3	0
11	1	1	1	0
12	1	1	1	0
13	2	2	2	0
14	12	11	11	0
15	4	4	3†	1
16	5	4	4	0
17	1	1	1	0
18	1	1	1	0
19	1	1	1	0
20	1	1	1	0
21	1	1	1	0
22	2	2	0	2

* 2 others doubtful.
† 1 other doubtful

ence of added water by the freezing-point. In the case of 2 samples (25, 26), having a color reaction of N-4 (pH 6.82 to 6.90) and of 2 samples (27, 28) with a reaction of N-5 (pH 6.90 to 6.98), the decreased acidity was due in every case to garget, the determination of the freezing-point showing the milks to be entirely normal in water content. For further details regarding the reaction of milk to udder infection see page 351.

3 The number of dairies furnishing the 570 samples of milk examined was 46. In the case of 16 dairies, the milks showed a

subnormal or decreased acid reaction by the brom-cresol purple test. Three-fourths of these subnormal samples came from a few herds. It was found that in one of these the milk was being watered regularly and some of the milk from the other herds gave evidence of severe mastitis. Table II shows the number of milks of subnormal reaction in the case of the 16 herds and indicates also the total number of examinations and the number of times the samples were found watered and, in addition, the cases where garget was present.

It is seen that in the case of the 16 herds, 58 examinations of milk were made; in 52 samples, the reaction was found to be subnormal or of decreased acidity In these 52 cases, 37 showed clear evidence of watering by the depression of the freezing-point and 3 others were so close to the border line as to be open to a strong suspicion of being watered. There were three herds in which persistent addition of water was shown.

4. The 6 samples, 23–28, were drawn from the udders under our direct supervision Four of these showed a subnormal reaction, owing to the presence of garget, and the freezing-point test shows that the percentage of water is not excessive. A complete chemical analysis of these four samples would undoubtedly show an abnormal composition according to our work (page 350)

Relation Between the Reaction of Milk and the Bacterial Content.

Milks showing a reaction above normal acidity, as indicated by giving with brom-cresol purple solution a lighter color, were examined for their bacterial content. Of the 570 samples examined, 16 gave a lighter color than normal, of which 11 were found by Miss Mildred C. Davis, the City Bacteriologist, to contain over 10,000,000 bacteria per cc. by the direct-count method[3] In the case of two of the other samples, the light color was due to high milk-fat content, bacteria not being present in large numbers

A further study was made at the Laboratories of the Department of Health of New York City through the courtesy of the Director, Dr. Wm. H. Park. Of the 11 samples found showing a light color with brom-cresol purple solution, four contained

over 1,000,000 bacteria per cc. by the plate-count method and six showed high percentage of milk-fat with low bacterial content.

SUGGESTIONS.

It has been stated already that the main source of weakness in the application of this method is the observation of the shade of color given by the sample of milk with brom-cresol purple solution. It is, therefore, important that, before one attempts to use the method in practical application, some special work be done in a study of the shades of color of the brom-cresol purple solution in milk under a great variety of conditions   For example, taking some fresh normal milk of average composition, that is, with 3 to 4 per cent of milk-fat, a portion is treated with brom-cresol purple solution in the manner described (page 361), and then other portions are treated by the addition of definite amounts of 0 1 N alkali, just as in the preparation of the standard color series (page 363), and other portions by 0 1 N lactic acid, while other portions are diluted with definite amounts of water, and others are skimmed, and others have cream added to them. Also the results of the action of definite amounts of formaldehyde added to portions of the milk should be studied; and also the effects of the addition of varying amounts of sodium bicarbonate, borax, etc   Portions of milk heated to various temperatures are similarly studied. A similar complete study should be made with different samples of normal milk until one is able to distinguish different shades of color so far as they have a meaning in practical applications of the test.

It should be emphasized here again that the application of the brom-cresol purple test is not to be regarded as final but only as preliminary and suggestive. Its chief value is to be found in the fact that, when properly used, it will greatly minimize the work involved in official milk inspection, because it will point in most cases directly to the milks that are abnormal and, therefore, indicate which samples need further detailed work to confirm or disprove the suspicion aroused by the result of the preliminary test.

SUMMARY.

The method described as a means for the preliminary detection of abnormal milks is based upon the use of the dye, dibrom-ortho-cresol-sulfon-phthalein, the name being abbreviated to brom-cresol purple One drop of a saturated water solution is mixed with 3 cc. of milk and the color is observed. Normal fresh milk gives a grayish-blue color   The production of a darker or lighter color serves to awaken suspicion in regard to the normal character of the milk   The color is made lighter by acids, formaldehyde, and also by heating above the usual point of pasteurization   The color becomes deeper blue in the case of milk from diseased udders, watered milk, skimmed milk, and milk containing added alkaline salts. In the inspection of milk, a sample is taken for further detailed examination in the laboratory if the color is sufficiently lighter or darker than normal to indicate the probability of some abnormal condition

The method has been applied and results are reported for 570 samples of market milk. Watered milk was detected and also milk containing excessive numbers of leucocytes   A standard of colors can be prepared by which comparison can be made and conclusions more easily reached as to the normality or abnormality of the samples examined.

A METHOD FOR THE DETERMINATION OF THE KEEPING QUALITY OF MILK.

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INTRODUCTION.

Keeping quality or keeping power is an expression used to indicate the length of time milk remains sweet and otherwise palatable and suitable for direct consumption. This is obviously an important factor in estimating the commercial value of market milk, since milk that is sour or otherwise unpalatable is comparatively valueless for direct use, however rich it may be in fat and other solids.

Various methods have been proposed for measuring the keeping quality of milk but these have been found unsatisfactory in actual practice. Therefore, a method which can be utilized to furnish consistent results in measuring, even though only approximately, the keeping quality of different milks is needed.

Proposed Method.

We have found that the brom-cresol purple test (see page 359) can, with simple modifications in technique, be applied to the measurement of certain factors affecting the keeping power of milk. In applying the test for this purpose, the test-tubes and pipettes must be sterilized before use and the milk in the test-tubes must be incubated for a stated length of time at a definite temperature. Examination of the milk after incubation furnishes evidence in respect to the keeping power of milk as shown by one or more of several possible changes that may take place in the milk. Such changes can be divided into two classes, first, those affecting the color of brom-cresol purple, which show a

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change of reaction in the milk due to the production of acid or less often to the formation of alkali salts, and, second, other accompanying or succeeding changes, such as curdling of the milk due to coagulation of casein, digestion of casein, changes in the character of the coagulated or curdled milk, production of gas, and the development of abnormal odor and taste.

Non-Germicidal Effect of Brom-Cresol Purple Solution.

In order to be of value as a means of measuring the keeping quality of milk by the reaction, it is essential that the brom-cresol purple solution should not, under the conditions used, show any germicidal effect sufficient to interfere with the growth of bacteria in milk. In order to test this fundamental requirement, pure cultures of *Bacterium lactis acidi* were added to freshly pasteurized skim-milk; one portion of this was treated with brom-cresol purple solution and both portions were incubated at 20°C. At intervals the brom-cresol purple test was applied to samples taken from the incubated portion of milk containing none of the indicator, and comparison was made with the portion to which brom-cresol purple had been added at the start. Also samples of the two portions of milk were titrated with alkali These tests were made many times with different milks, but in no case was there observable any difference in behavior. The same tests were also applied in numerous cases to two portions of a milk undergoing the process of natural souring, using both unheated and pasteurized milk, without showing any difference. The results all go to show that the brom-cresol purple solution has no germicidal effect under the conditions used. However, it is advisable to take precaution to use for this test only brom-cresol purple that is wholly free from the odor of phenol or cresol, as suggested by Clark and Lubs [1] In our experience it is not difficult to obtain this.

Production of Acid.

In considering the application of the brom-cresol purple test to the measurement of the keeping quality of milk as shown by the formation of acid in milk, we will present the subject under

[1] Clark, W M , and Lubs, H A , *J. Agric. Research*, 1917, x, 105.

the two heads, localization of acid production and degrees of acid production.

Localization of Acid Production.—In the natural souring of milk standing undisturbed, the formation of acid rarely proceeds uniformly through the body of the milk but is largely localized, especially in the earlier stages of the process   Acid is usually first formed in appreciable amount at the upper surface next the cream layer, or less often in the layer at the bottom of the container, or it may appear in some cases simultaneously in both the top and bottom layers   Less frequently it may start in the layer of milk next the side walls of the container   •

When acid is formed first at the upper surface of the milk, it is probably due to the fact that the organisms are enmeshed and carried upward with the rising fat-globules and are thus concentrated in the upper layer   The bacteria left in the body of the milk after the rising of the fat-globules would tend, under the downward pulling effect of gravity, to settle at the bottom of the container.   Generally, the number carried up is apparently greater than that carried down   Such a concentration of bacteria in the top or bottom layer of the milk would have the effect of making the brom-cresol purple test more sensitive as a result of more rapid formation of acid   The effect of acid development is more commonly shown first in the upper layer, though sometimes in the lower or less often in the side layer.   But whether it starts at the top or bottom or side, the process of acid production works from the starting area or areas through the main body of the milk

Degrees of Acidity —It would be desirable, if it were possible, to distinguish different degrees of increasing acidity by preparing a color standard representing different values of hydrogen ion concentration, similar to the method described on page 363 for determining the approximate hydrogen ion concentration of milk when its acidity is less than that of normal milk.   This is impossible for several reasons and especially because, as pointed out above, the production of acid is localized and not distributed uniformly through the body of the milk   However, we have found that it is possible, with some experience, to distinguish readily not less than four degrees or stages of acidity by changes of color, varying from the grayish-blue observed with normal

fresh milk to a pure yellow occurring in milk sufficiently sour to undergo coagulation, which occurs at about pH 4.65. These four stages or degrees of acidity can be distinguished by the following description

(1) The first stage or beginning of acid production (A_1) is indicated by the first sign of change from the grayish-blue color of normal milk to a lighter shade observable in any portion of the milk. This is most often distinguishable at the upper surface of the milk just under the cream layer, though it may occur at the bottom layer of the milk or less frequently at the side walls of the containing vessel

(2) The second stage (A_2) shows distinct acid production and is indicated when the milk in a test-tube gives evidence of more extensive and marked change of color than in case of A_1 The main body of the milk, however, still retains a grayish-blue color more or less interspersed with, but predominant over, yellowish or greenish-yellow shades. The prevailing color may be bluish or a dull shade of bluish-green

(3) The third stage (A_3) shows marked acid production and is indicated when the color of the milk in the test-tube appears greenish to greenish-yellow; the yellow is predominant through the body of the milk, though not complete, but is more or less interspersed with shades intermediate between dull green and yellow.

(4) The fourth stage (A_4) of acid production is easily observable, since the color is a pure, fairly uniform yellow, free from every trace of bluish or greenish tints. The curdling of the milk usually occurs at this stage and is generally, though not always, readily seen

It can easily be understood that these divisions are somewhat arbitrary and not always capable of sharp separation but they afford a practical basis for differentiating milks, furnishing a test which greatly exceeds in delicacy and ease of application the usual titration methods With experience in distinguishing shades of color, it is easily possible to carry the division of classes further if desired; but usually the four broad classes described above suffice for most purposes.

Other Changes in Milk.

Changes other than those produced by acid formation can also be observed, and to these attention will be briefly called. Such changes may occur only after somewhat prolonged incubation in the case of good market milks, but they appear more quickly in the case of milks which have been drawn more than 24 hours before incubation, or in the case of milks drawn under unfavorable conditions as to cleanliness and not kept at a sufficiently low temperature. It should be stated here that while these changes have been studied by bacteriologists, it is essential that they be given special attention and further study under the conditions of the proposed test.

1. Production of alkali during incubation is shown by decreased acidity and is indicated by increase of depth of the grayish-blue color given with normal milk by the brom-cresol purple solution.

2 Digestion of casein is observable just below the cream layer and is indicated by the appearance of a layer of more or less clear solution.

3. Gas production is easily observed, indicating the presence of gas-producing organisms. This test is especially valuable in connection with milk to be used for cheese-making.

4 The contraction or shrinking of the curd or coagulated casein into a smaller mass is easily seen when it occurs. This is accompanied by the separation of more or less clear whey.

5. Any abnormal odor or taste is readily ascertained by any one having well developed senses of smell and taste. Such abnormal conditions have been noticed in our experience only in the case of milks which had shown marked change in reaction as indicated by the color given with brom-cresol purple solution.

RESULTS OF APPLICATION OF THE METHOD.

In applying the brom-cresol purple test to the measurement of acid production in relation to the keeping quality of milk, two separate series of experiments will be presented   In the first series, the samples used were taken from individual cans of milk as delivered by producers at the two collecting stations handling the supply of the city of Geneva.   In the second series,

the samples were obtained from the regular milk supply of New York City.

1 The Geneva Samples.—In applying our method to 389 samples obtained in Geneva, we have had the cooperation of the city bacteriologist, Miss Mildred C Davis, who classified the samples into groups by microscopical examination, using the direct-counting method [2]

The results are summarized in Table I.

In interpreting the results of the microscopical examination with reference to the fitness or keeping quality of milk for domestic use, milks in Class I are regarded as excellent, in Class II as satisfactory, in Class III as unsatisfactory, and in Class IV as

TABLE I

Comparison of Results of Brom-Cresol Purple Test with Classification by Microscopical Examination

Class	No of individual bacteria per cc of milk	No of samples examined	No of samples changing color	No of samples not changing color	Milk showing good keeping quality	Milk showing poor keeping quality
					per cent	*per cent*
I	Below 350,000	283	41	242	85 5	14 5
II	Between 350,000 and 1,000,000	21	6	15	71 5	28 5
III	"　　1,000,000 and 10,000,000	52	33	19	36 5	63 5
IV	Over 10,000,000	33	27	6	18 0	82 0

very unsatisfactory. While there is a general correspondence between the results obtained by the microscopical examination and by the brom-cresol purple test, the agreement is not complete In Class I, representing milk of excellent quality by microscopical examination, 242 samples out of 283 show no change by the brom-cresol purple solution, thus confirming the results of the microscopical examination; but 41 samples out of the 283, or 14.5 per cent, show sufficient increase of acidity to be detected by the brom-cresol purple solution In Class II, of the 21 samples graded as satisfactory by microscopic examination, 6 samples, or 28.5 per cent, show increase of acidity with brom-cresol purple solution. In Class III, 52 samples are graded as unsatisfactory

[2] Breed, R S , *New York Agric Exp Station, Techn Bull 49,* 1916.

by the microscopic method, while 19 samples, or 36.5 per cent, fail to show increased acidity. In Class IV, 33 samples are graded as very unsatisfactory by the microscopical method, but of these there are 6, or 18 0 per cent, which show no increase of acidity   These observed differences of interpretation in the application of these two methods to the determination of keeping quality in milk are what might be expected under the conditions and are easily explained. The brom-cresol purple test is here applied to detect increase of acidity while the microscopical examination includes all kinds of bacteria and not merely those capable of producing acid   It is obvious that in the case of bacteria producing no acid or only very small amounts under the conditions of the test, the brom-cresol purple test would not be expected to apply as it does in the case of marked acid producers.

2. The New York City Samples.—These samples were obtained in the regular inspection work of the city milk supply, through the courtesy of Dr. Wm. H. Park, Director of the Laboratories of the Department of Health. We are indebted also to Dr. Hazel Hatfield for the work done in making the bacteriological examinations of the samples. There were examined 220 samples of unheated or raw milk and 186 of pasteurized milk. The bacteriological examination was made by the official plate method after incubation at 37°C. The tests with brom-cresol purple solution were made in all cases on samples incubated at 18°C. for 24 hours   This temperature is higher than that found in efficient household refrigerators, but it may be regarded as representing approximately the average temperature at which milk is kept after delivery to the consumer

The division into classes on the basis of bacterial content has been carried farther than in case of the Geneva milks, providing fifteen different divisions as shown in Table II.

The results with the raw milks examined in New York City show, in general, that when the numbers of bacteria increase, there is an increase in the percentage of samples showing increase of acidity. Milks with high bacterial content usually show poor keeping quality by the brom-cresol purple test, while those containing small numbers of bacteria generally show good keeping quality by the color test. However, some milks with fairly high

TABLE II

Results of Examination of New York City Milks

Class	R = raw P = pasteurized	No of colonies developed per cc of milk	No of samples	No of samples showing change of reaction	No of samples not showing change of reaction	Milk classed as poor by test	Milk classed as good by test
						per cent	per cent
I	R	Below 1,000	0	0	0	0	—
II	"	Between 1,000 and 5,000	4	0	4	0	100
III	"	" 5,000 " 10,000	6	0	6	0	100
IV	"	" 10,000 " 20,000	4	3	1	75	25
V	"	" 20,000 " 30,000	10	5	5	50	50
VI	"	" 30,000 " 60,000	17	14	3	82 4	17 6
VII	"	" 60,000 " 100,000	23	22	1	95 7	4 3
VIII	"	" 100,000 " 150,000	25	20	5	80	20
IX	"	" 150,000 " 250,000	57	54	3	94 7	5 3
X	"	" 250,000 " 400,000	16	14	2	87 5	12 5
XI	"	" 400,000 " 600,000	16	16	0	100	0
XII	"	" 600,000 " 1,000,000	20	20	0	100	0
XIII	"	" 1,000,000 " 1,500,000	10	9	1	90	10
XIV	"	" 1,500,000 " 3,000,000	7	7	0	100	0
XV	"	Over 3,000,000	5	5	0	100	0
I	P	Below 1,000	4	0	4	0	100
II	"	Between 1,000 and 5,000	16	1	15	6 2	93 8
III	"	" 5,000 " 10,000	9	1	8	11	89
IV	"	" 10,000 " 20,000	32	0	32	0	100
V	"	" 20,000 " 30,000	32	2	30	6 2	93 8
VI	"	" 30,000 " 60,000	28	3	25	10 7	89 3
VII	"	" 60,000 " 100,000	20	5	15	25	75
VIII	"	" 100,000 " 150,000	15	9	6	60	40
IX	"	" 150,000 " 250,000	13	12	1	92 3	7 7
X	"	" 250,000 " 400,000	4	2	2	50	50
XI	"	" 400,000 " 600,000	5	5	0	100	0
XII	"	" 600,000 " 1,000,000	3	3	0	100	0
XIII	"	" 1,000,000 " 1,500,000	5	5	0	100	0
XIV	"	" 1,500,000 " 3,000,000	0				
XV	"	Over 3,000,000	0				

bacterial content show good keeping quality, while some with a low content show poor keeping quality, by the brom-cresol purple test.

With pasteurized milks we obtain similar results, except that a large proportion of the milks with the higher bacterial content show good keeping quality by the brom-cresol purple test.

The results obtained with the New York City milks are not properly comparable with those obtained with the Geneva milks, especially for two reasons. In the first place, the two methods of obtaining the bacterial content, Breed's direct-counting method and the so-called official plate method, do not give results sufficiently comparable for our purpose. In the second place, the New York City milks average probably not less than 24 hours old when the samples are used for laboratory work, while the Geneva samples are not more than 6 to 16 hours old.

In the summary of the results here presented, we do not give the varying degrees of acidity developed on incubation but only the general fact of an increase. Data in greater detail are being collected.

Additional Work.

While some observations have been made on the relation of the other factors to keeping quality, much additional work remains to be done along the following lines: (1) Digestion of casein, (2) production of alkali, (3) production of gas, (4) taste and odor, (5) relation of age of milk to temperature and length of time of incubation.

SUMMARY.

1. Brom-cresol purple can be used to measure approximately and relatively the keeping quality of milk. The test is applied in the manner described in the article preceding (page 357), with the modification that the pipettes and test-tubes used are sterilized before sampling the milk, and, further, the samples of milk in the test-tubes must be incubated a given time at a given temperature (usually 18° to 20° C.). The milk is examined for changes of color at 24 hour intervals The main factor shown by this test as related to keeping quality is production of acid, but

additional factors to be observed are coagulation of casein, digestion of casein, production of alkali, production of gas, development of abnormal odor and taste

2. In showing the development of acidity, four stages of. progress are distinguishable through change of color, varying from the grayish-blue of brom-cresol purple in normal milk to a final clear yellow, the intermediate stages showing mixtures of color. In comparing this test with the bacterial count, it is found that in general large numbers of bacteria and increase of acidity are in fair correlation.

3. The other factors related to keeping quality, such as digestion, gas, alkali production, and abnormal odor and taste, are readily observable, but frequently not until after 24 hours of incubation.

EXTRACTION AND CONCENTRATION OF THE WATER-SOLUBLE VITAMINE FROM BREWERS' YEAST.*

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New Haven)*

In making experiments to determine the nutritive value of individual proteins it is necessary to employ a source of the water-soluble vitamine in the synthetic diet   Heretofore it has been customary to feed some natural product such as protein-free milk, wheat embryo, or yeast to supply the necessary amount of this vitamine. All such products, however, contain relatively considerable quantities of nitrogenous substances, including proteins, hence there is always a question as to whether, or not, these so supplement deficiencies in the protein being tested as to give more or less misleading results.   Such criticisms are not entirely unjustified because the nitrogen supplied together with the vitamine may be equal to 10 to 12 per cent of the nitrogen fed.   Since some of such nitrogen belongs to protein and most of this to amino-acids identical with those contained in the protein being tested there is little reason to believe that the results of the experimental feeding are seriously affected.   Nevertheless there is always a possibility that this may happen and we have had a feeling that some of our results may have been affected to a noticeable extent.   A concentrated preparation of the water-soluble vitamine would also be of value for feeding experiments with inorganic salts as well as with individual carbohydrates. With a view to producing such a preparation we have attempted to concentrate the water-soluble vitamine in a fraction of yeast

We have long known that the water-soluble vitamine is insoluble in absolute alcohol, hence it seemed probable that by

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fractionally precipitating the aqueous extract with alcohol this might be concentrated in a fraction and thus be obtained together with a comparatively small proportion of the other constituents of the yeast.

The first step towards this end was the preparation of an aqueous extract which should contain as much of the vitamine and as little of the other constituents of the yeast as possible. Almost all investigators who have attempted to separate the water-soluble vitamine from yeast have apparently considered it necessary to bring into solution as much as possible of the contents of the yeast cells. To accomplish this they have generally allowed the yeast to undergo autolysis, whereby a considerable part of the protein, as well as of other constituents of the yeast, is converted into water-soluble products largely of unknown character.

If the protein in the living yeast could be coagulated by boiling water before undergoing any autolytic change, and the water-soluble vitamine be simultaneously extracted, the conditions for a concentration of this vitamine in a fraction of the water extract would be greatly simplified

EXPERIMENTAL.

Several liters of fresh bottom yeast were obtained directly from a brewery, and immediately diluted with ice water. After centrifuging, the sediment was washed twice more in the same way. The moist, washed yeast weighed 264 gm., equal to 48 gm. dried at 107°. This was stirred gradually into 1 liter of boiling distilled water containing 10 cc. of 1 per cent acetic acid. After boiling for about 2 minutes the solids were separated from the extract with the centrifuge. The residue was washed once by boiling with 0 01 per cent acetic acid and, after centrifuging, the extracts were united and concentrated to 500 cc. This concentrated extract contained 8.14 gm of solids, equal to 17 1 per cent of the dry yeast, and 0.666 gm of nitrogen, equal to 14 4 per cent of the original yeast nitrogen, or to 8 18 per cent of the solids of the extract

Although this extract contained less than one-fifth of the yeast solids it contained nearly all the water-soluble vitamine.
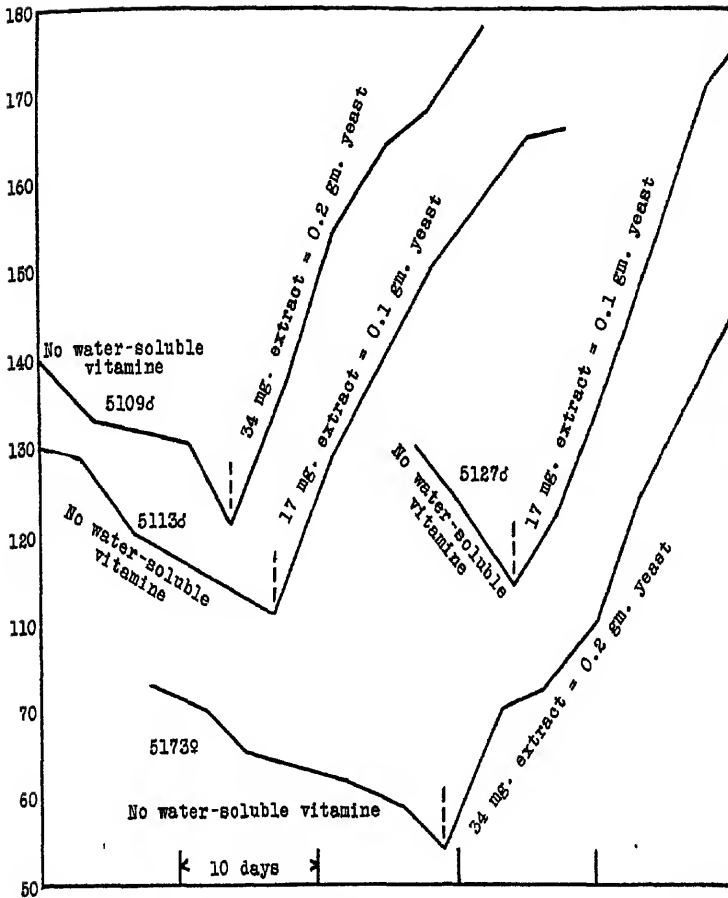
CHART I   Young rats declining on a diet free from water-soluble vitamine recover when the solids extracted from live yeast by boiling water are added in a quantity equal to 0 1 to 0 2 gm. of the original yeast

Chart I (see Rats 5113 and 5127) shows that daily doses of 17 mg. of the solids of this extract promoted the recovery and rapid growth of young rats declining on a diet free from the water-soluble vitamine, but which in other respects was adequate When 34 mg. of the yeast extract were fed (see Rats 5109 and 5173) the rate of gain was no greater, hence it appears that the smaller dose supplied as much of the water-soluble vitamine as

these animals needed. These doses were equivalent to only 0.1 and to 0.2 gm respectively of the original dried yeast Usually 0.1 gm of dried yeast is not sufficient to promote such a vigorous gain of weight as these rats showed, hence we conclude that this boiling water extract contained nearly all the water-soluble vitamine of the yeast from which it was derived.

When we found that this aqueous extract was so rich in the water-soluble vitamine, a similar extract from a larger quantity of fresh bottom yeast was subjected to fractional precipitation by alcohol. After washing with ice water, the 4 5 kilos of the moist yeast, equal to 830 gm dried at 107°, were stirred slowly into 10 liters of boiling water containing 0 01 per cent of acetic acid. After boiling about 5 minutes the extract was readily filtered through folded soft papers The residue was washed once by boiling with 5 liters of 0.01 per cent acetic acid and the washings, united with the main extract, were concentrated to 2 liters This contained 140 gm. of solids, equal to 16 9 per cent of the dry yeast, and 12 02 gm. of nitrogen, equal to 13.7 per cent of the original yeast nitrogen, or to 8.6 per cent of the solids of the extract. These are substantially the same proportions as were found in the preceding experiments.

The concentrated extract was then poured into 3 liters of 93 per cent alcohol, making the alcoholic content of the mixture about 52 per cent by weight. The flocculent precipitate, Fraction I, which separated, when washed with 52 per cent alcohol, digested with absolute alcohol, and dried over sulfuric acid, formed a nearly white powder, equal to 35.9 gm. dried at 107°. This fraction formed 4.3 per cent of the dry yeast or 25 6 per cent of the solids of the water extract. It contained 1.72 gm. of nitrogen, equal to 1 9 per cent of the original yeast nitrogen, or to 4.8 per cent of the precipitate. Its ash content was 47.15 per cent.

The filtrate and washings from Fraction I were concentrated to 300 cc. and poured into 1,960 cc of 93 per cent alcohol, making the alcoholic content of the mixture about 79 per cent by weight. The precipitate, Fraction II, thereby produced was washed once with 79 per cent alcohol, and then twice dissolved in about 100 cc. of water, and reprecipitated by pouring into enough alcohol to make the alcoholic concentration 90 per cent by weight. After

digesting under absolute alcohol and drying over sulfuric acid a light-colored, friable product was obtained equal to 51.8 gm. dried at 107°, or to 37 per cent of the solids of the extract, or to 6.2 per cent of the dried yeast. This contained 7.5 per cent of nitrogen, equal to 4 5 per cent of the nitrogen of the original yeast, and 10 65 per cent of ash.

The united solutions from Fraction II were concentrated to a small volume and poured into enough absolute alcohol to make the alcoholic concentration of the mixture 90 per cent by weight. The precipitate which separated, Fraction III, was dissolved in 100 cc. of water and again precipitated by alcohol at 90 per cent. Since this precipitate was gummy it was again dissolved in about 30 cc of water and the solution poured into 500 cc. of absolute alcohol. The still gummy precipitate was digested with absolute

TABLE I

	Solids	Nitro-gen	Solids		Nitrogen		Ash of fraction
			Of yeast	Of water extract	Of fraction	Of yeast	
	gm	*gm*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Water extract	140 0	12 0	16 9	100 0	8 6	13 72	21 70
Fraction I	35 9	1 7	4 3	25 6	4 8	1 94	47 15
" II .	51 8	3 9	6 2	37 0	7 5	4 45	10 65
" III	13 9	1 8	1 6	9 9	13 1	2 06	15 90
" IV	36 1	3 9	4 4	25 8	10 8	4 45	15 90

alcohol and dried over sulfuric acid. Fraction III weighed 13.85 gm dried at 107°, equal to 1.6 per cent of the original dried yeast, or to 9 9 per cent of the solids of the water extract, and contained 1 81 gm. of nitrogen equal to 13.1 per cent of the fraction, or to 2.06 per cent of the yeast nitrogen. Its ash content was 15.9 per cent.

The strong alcoholic solutions from Fraction III were united and concentrated to small volume Owing to the gummy character of the dissolved solids these were preserved in strong alcohol. This solution contained solids, Fraction IV, equal to 36.1 gm. dried at 107°, equivalent to 4 4 per cent of the original yeast or to 25.8 per cent of the solids of the water extract. It contained 10.83 per cent of nitrogen, equivalent to 4.5 per cent of the yeast nitrogen, and 15 9 per cent of ash. The results of this fractionation are summarized in Table I.

If the water-soluble vitamine was wholly concentrated in any one of these fractions quantities of each equivalent to the original yeast from which it was derived should be as effective as the latter in promoting the recovery of young rats declining on a diet free from this accessory. As 0.2 gm. of the entire yeast has proved to be sufficient for this purpose, and 0 1 gm. usually to be insufficient, these fractions were mixed with starch in such proportion as to make the mixture contain the same percentage of the fraction as did the original yeast Thus 0.2 gm. of the starch-fraction mixtures contained of Fraction I 8.6 mg , II 12.4 mg., III 3 2 mg., IV 8 8 mg. Since Fraction II formed a larger percentage of the yeast than did any of the other fractions the absolute amount of each of these fractions in the respective starch mixtures was less than that of Fraction II. The feeding experiments to be described consequently do not afford a strict comparison of the efficiency of the fractions relatively to one another. They do, however, enable us to determine in which fraction the greater part of the total water-soluble vitamine of the yeast was concentrated. Charts II, III, and IV show the results of such feeding experiments. Chart II (Rats 5191, 5199, and 5291) shows that no appreciable gain of weight was made when doses of Fraction I equivalent to 0.2 gm. of yeast were fed daily. That this fraction was not wholly free from the water-soluble vitamine is shown by the slight gains made by Rats 5191 and 5291 after doubling the dose. When Rat 5199 was given daily doses of Fraction II corresponding to 0.2 gm of yeast, it gained weight rapidly. Chart III shows that Rats 5116 and 5289 which received daily doses of Fraction II equivalent to 0 2 gm. of yeast gained weight rapidly. Rat 5116 when given 0.2 gm. of yeast grew no faster Rat 5198 which received a daily dose of Fraction II equivalent to only 0 1 gm of yeast made a fairly rapid gain of weight although only 6 2 mg. of the fraction were fed. Chart IV shows that Rats 5054 and 5288, to which doses of Fraction III equivalent to 0 2 gm. of yeast were given each day, and Rats 5039 and 5131, to which similar doses of Fraction IV were given, gained so little weight that it is evident that neither of these fractions contained as large a proportion of the water-soluble vitamine of the original yeast as did Fraction II. The slight gain of weight made by the rats having Frac-
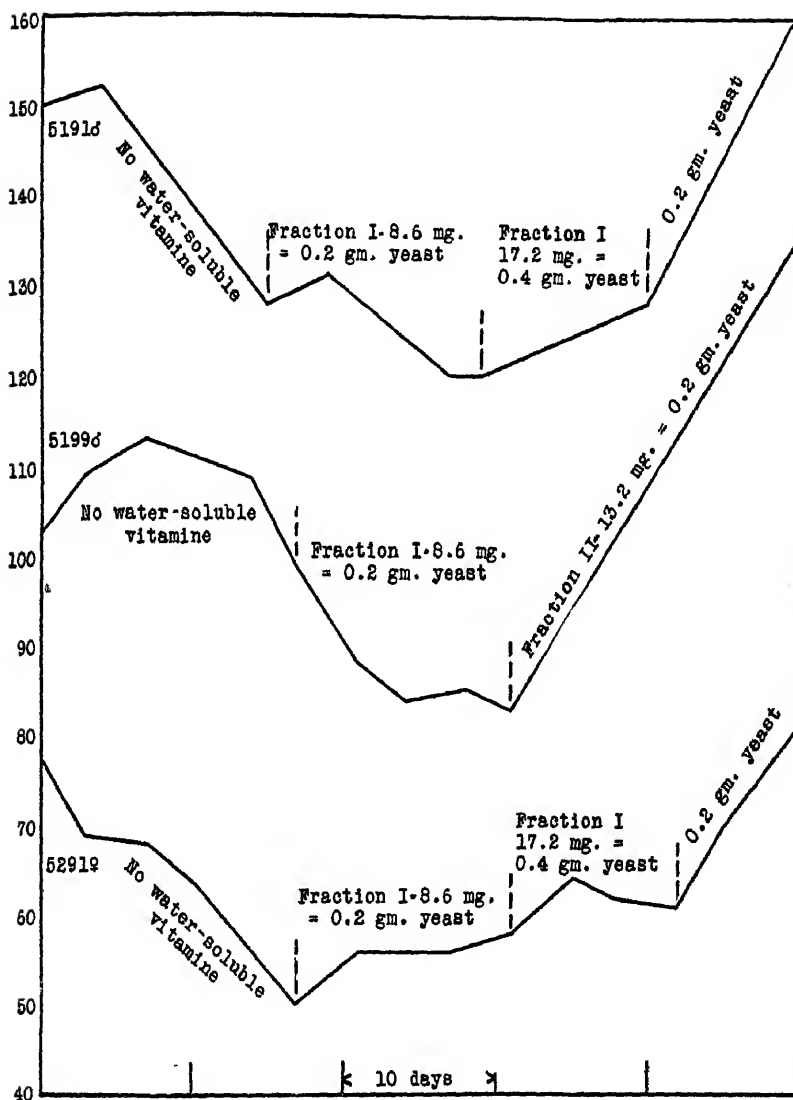
CHART II   Young rats after declining on a diet free from water-soluble vitamine fail to recover when quantities of Fraction I, equivalent to 0 2 gm of yeast are added daily to their diets, but recover when given similar proportions of Fraction II or 0 2 gm. of yeast   Even doubling the quantity of Fraction I caused very little gain in weight.
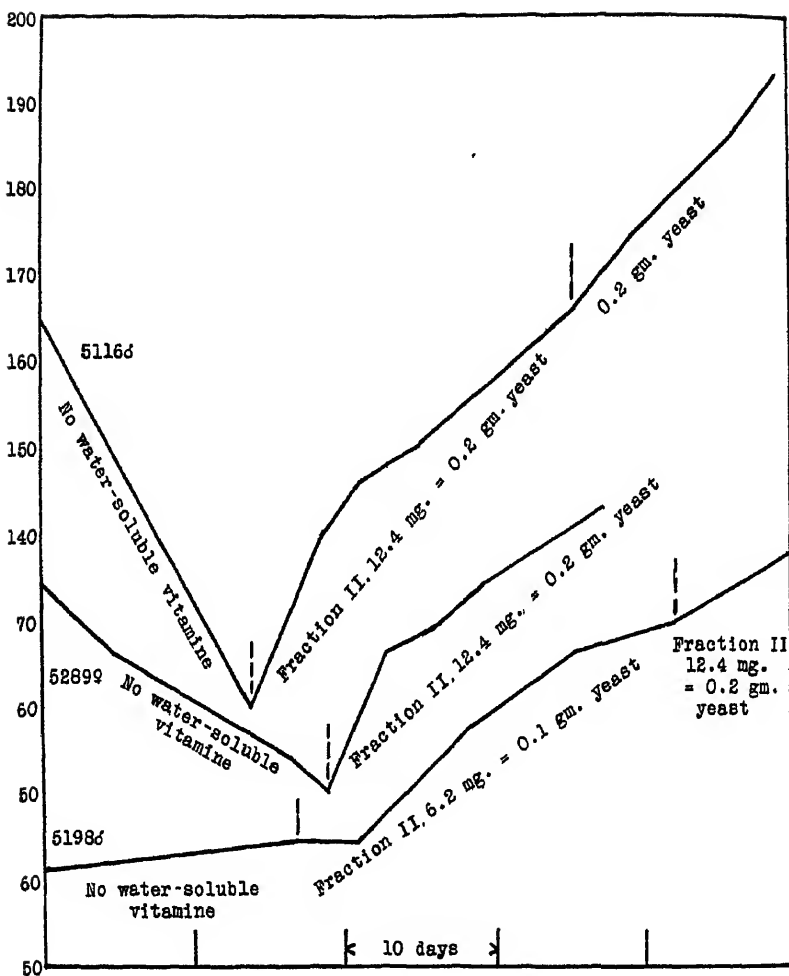
CHART III. Young rats declining on a diet free from water-soluble
vitamine recover when given quantities of Fraction II equivalent to 0.2
or 0 1 gm. respectively of yeast   When 0 2 gm. of yeast was given to Rat
5116 instead of Fraction II it did not gain any faster.
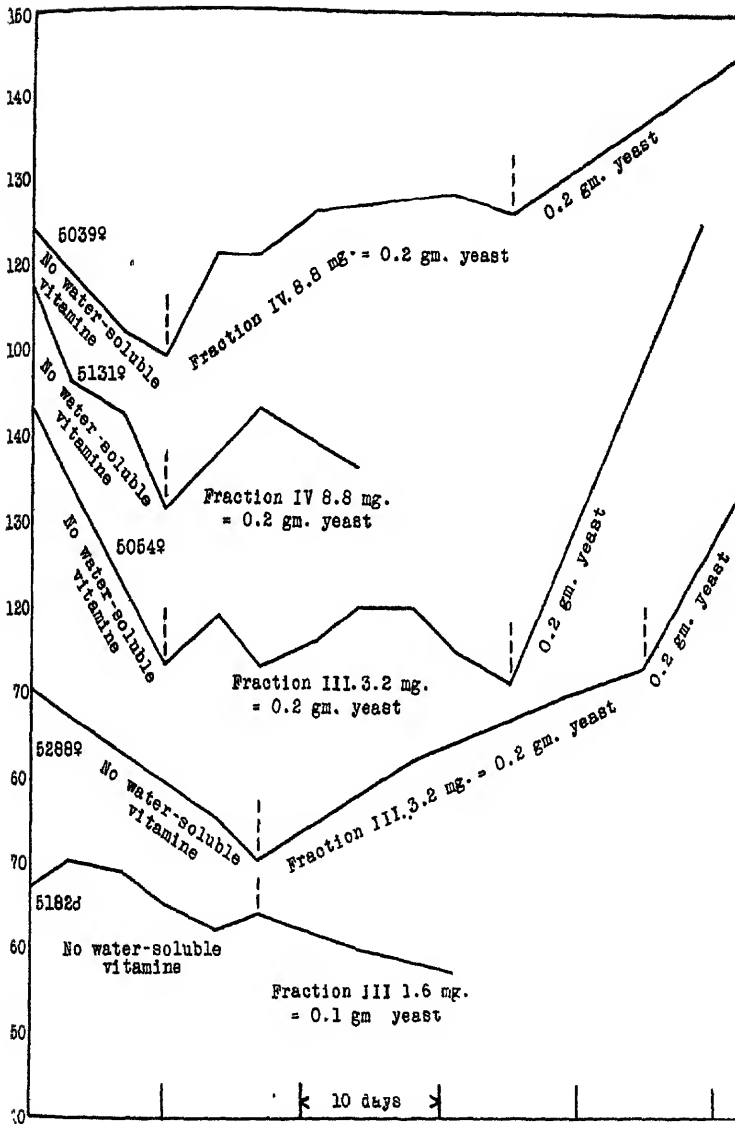
CHART IV Young rats declining on a diet free from the water-soluble vitamine fail to recover when given quantities of Fractions III or IV equivalent to 0 2 gm of the original yeast. Recovery is prompt when 0 2 gm of yeast is given.

tions III and IV indicates, however, that these fractions contained some of the water-soluble vitamine. In view of the fact that daily doses of only 3.2 mg. of Fraction III enabled Rat 5288 to make a relatively considerable gain of weight shows that this fraction contained, weight for weight, as much, if not more, of the water-soluble vitamine as did Fraction II. However, since Fraction III amounted to only 1 6 per cent of the yeast it contained only a relatively small part of the total water-soluble vitamine. There can be no question therefore that Fraction II contained most of the water-soluble vitamine originally present in the yeast.

Having thus found that the greater part of the water-soluble vitamine can be concentrated in about 6 per cent of the yeast solids it is now possible to supply enough of this vitamine for normal nutrition without introducing such relatively large quantities of nitrogenous substances of unknown nature as have heretofore usually been necessary. Whether the method of fractionation here described is superior to precipitation by adsorption on Lloyd's[1] reagent remains to be determined, but it would seem as if our Fraction II offered advantages for the further study of many problems concerning the water-soluble vitamine According to Seidell, Lloyd's reagent adsorbs 4.5 per cent of the nitrogen from the autolyzed yeast filtrate simultaneously with the vitamine, whereas our Fraction II contained 4.46 per cent of the total yeast nitrogen or 31.8 per cent of the nitrogen of the yeast extract.

As this Fraction II is easily prepared in large quantities it will certainly afford a better crude material for further study than does the autolyzed yeast filtrate which contains a large proportion of the products of autolysis derived from the yeast protein which, as our experiments show, are not concerned in the activity of vitamine preparations made from yeast The chief advantage of the procedure here described lies in the preparation of the aqueous extract, for by avoiding autolysis the proportion of water-soluble constituents of the extract is reduced to a minimum while the quantity of water-soluble vitamine in the extract is apparently not diminished

[1] Seidell, A., *J. Biol Chem* , 1917, xxix, 145.

It will be a matter of interest to learn more than is now known about the chemical nature of the constituents of this water extract of the living yeast, not only in connection with further attempts to concentrate the water-soluble vitamine, but also as a contribution to the chemistry of cytoplasm

As yet we have made only a preliminary examination of this interesting mixture. That it consists chiefly of nitrogenous substances is indicated by its content of about 8.5 per cent of nitrogen. Proteins are not present in amounts detectable by saturating with ammonium sulfate, or by potassium ferrocyanide and acetic acid. A biuret reaction has been obtained only by very carefully applying this test to the part precipitated by 52 per cent alcohol. Inorganic constituents are also abundant, because the ash forms over 20 per cent of the dry solids. The different types of nitrogen in the water extract in percentage of the total nitrogen are shown in Table II.

TABLE II

Types of Nitrogen in Per Cent of Total Nitrogen

	Humus N	NH₃ N	NH₂ N	Basic N	Purine N
Before hydrolysis	0	0	42	50	0
After "	0 64	1.56	60	50	18

The above figures indicate the presence of relatively large proportions of nucleic acid, amino-acids, and peptides, but as yet none of these has been isolated   Phosphatides are probably absent, because the ether extracts from Fractions II, III, and IV, the latter soluble in strong alcohol, failed to give any precipitate when poured into acetone

Fraction II which contains most of the water-soluble vitamine presents greater interest than the aqueous extract   This fraction is very soluble in water, its solution being distinctly acid to litmus. Relatively considerable quantities of alkali are needed to produce a neutral reaction to litmus and not a little more must be added before an alkaline reaction results.   Only a trace of a precipitate separates from the neutralized solution.

The unneutralized solution gives a large precipitate with lead acetate.   Barium chloride causes only a turbidity, but yields an

abundant precipitate when the solution is previously neutralized with sodium hydroxide. Silver nitrate does likewise.

Baryta solution gives a voluminous precipitate which contains about 25 per cent of the solids of the fraction and a relatively small part of its nitrogen. About 25 per cent more of the fraction is thrown out of the alkaline filtrate from the barium precipitate by silver nitrate. This precipitate, when thoroughly washed with baryta solution, contains nearly one-half the nitrogen of the fraction. The aqueous solution of Fraction II when acidified with sulfuric acid yields a very large precipitate with phosphotungstic acid. Mercuric chloride gives a precipitate; copper sulfate gives none. An aqueous or alcoholic solution of picric acid gives a precipitate if enough is added. Whether any one of the above precipitates contains some or all of the water-soluble vitamine remains to be determined.

Such preliminary observations indicate that a variety of substances are present in this fraction, but give no clue to the nature of the water-soluble vitamine. We have provided ourselves with several kilos of this active yeast fraction and are now engaged in a systematic study of its constituents

CRYSTALLINE SALTS OF URIDINPHOSPHORIC ACID.

By P A LEVENE

(From the Laboratories of The Rockefeller Institute for Medical Research)

Uridinphosphoric acid was previously described in form of its crystalline brucine and barium salts.[1] The identification of a nucleotide in form of its brucine salts was found not sufficiently reliable. The crystallization of the barium salt of uridinphosphoric is a slow and tedious process   Hence it was desirable to find such salts of the nucleotide which could be crystallized with readiness when one is in possession even of only a small quantity of material. In this communication are described several such salts; namely, the mono- and the diammonium salts of uridinphosphoric acid, the neutral lead salt, and the brucine salt prepared from the crystalline ammonium salt.

The neutral ammonium salt is readily prepared from the brucine salt and crystallizes as heavy, elongated prisms   In aqueous solution its optical rotation is $[\alpha]_D^{20} = +21.0$. It crystallizes with one crystal water.

The mono-basic salt crystallizes in form of long, prismatic needles   It is very soluble in cold and hot water, and in hot glacial acetic acid. In aqueous solution it has an optical rotation of $[\alpha]_D^{20} = +13 0$ The air-dry substance contains no crystal water

The neutral lead salt crystallizes in long needles, and was found very useful in obtaining pure uridinphosphoric acid when the original brucine salt had not been sufficiently purified.

The brucine salt was prepared in order to establish the constants of the compounds, since there was no conclusive evidence as to the absolute purity of the brucine salt obtained on fractionation of the mixed brucine nucleotides derived from the products of hydrolysis of nucleic acid

[1] Levene, P A , *Proc. Soc Exp Biol. and Med* , 1917, xv, 21, *J Biol. Chem* , 1918, xxxiii, 229.

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EXPERIMENTAL.

Di-basic ammonium salt was prepared by decomposing with aqueous ammonium hydroxide a solution of the brucine salt of uridinphosphoric acid in 35 per cent alcohol. The brucine salt of the mononucleotide was obtained either on acid or on ammonia hydrolysis of yeast nucleic acid.

The crude ammonium salt was dissolved in a minimum volume of hot water and to the solution hot methyl alcohol was added to very slight opalescence. On standing over night a deposit of heavy crystals formed. In subsequent experiments crystallization began immediately after addition of alcohol, if the solution was seeded with a crystal of the pure substance. The substance decomposed at 185°C (uncorrected).

0 0994 gm of the substance gave 0 1048 gm of CO_2 and 0 0486 gm of H_2O

0 1000 gm of the substance analyzed for Kjeldahl nitrogen estimation required 10 68 cc of 0 1 N acid

0 3000 gm of the substance gave 0 0870 gm of $Mg_2P_2O_7$

	Calculated for $C_9H_{19}N_4PO_9 + \frac{1}{2} H_2O$ per cent	Found per cent
C	29 11	28 75
H .	5 42	5 47
N	15 22	14 95
P . . .	8 44	8 08

The optical rotation of the substance was as follows.

$$[\alpha]_D^{20} = \frac{+\ 0\ 42 \times 100}{1 \times 2} = +21\ 0$$

Mono-basic ammonium salt was prepared in the following way: 2.0 gm. of the neutral salt were dissolved in 15.0 cc of glacial acetic acid, and to the hot solution hot ethyl acetate was added dropwise. Care was taken to wait with further addition until the precipitate forming on contact of ethyl acetate with the solution had disappeared After a slight opalescence was established, the solution was allowed to stand over night A crystalline sediment was found in form of balls consisting of long needles In subsequent experiments, if the solution in glacial acetic was seeded with the pure substance, crystallization began immedi-

ately. The air-dry substance contracted at 210°C. (corrected) and decomposed at 242°C. (corrected).

0.1000 gm of the substance gave 0 1142 gm of CO_2 and 0 0452 gm of H_2O.

0 1000 gm of the substance employed for Kjeldahl nitrogen estimation required for neutralization 8 8 cc of 0 1 n acid.

0 3000 gm. of the substance gave 0 0943 gm of $Mg_2P_2O_7$

	Calculated for $C_9H_{13}N_3PO_9$ per cent	Found per cent
C	31 66	31 14
H	.4 73	5 05
N	12 32	12 32
P	9 09	8 76

The optical rotation of the aqueous solution was as follows.

$$[\alpha]_D^{20} = \frac{+\,0\ 26 \times 100}{1 \times 2} = +\,13\ 0$$

Neutral lead salt was prepared in the following way: 2 0 gm. of the neutral ammonium salt were dissolved in 50 cc of water; to the solution 10 cc. of glacial acetic acid were added, and to the hot solution of the nucleotide a hot solution of neutral lead acetate was added. Immediately a gelatinous precipitate formed which, on boiling, disappeared nearly completely. The solution was filtered and seeded with a few crystals obtained from a test-tube experiment Crystallization in long needles began immediately. The crystals once formed are very little soluble in boiling water.

Dried to constant weight the substance had the following composition.

0 1070 gm of the substance gave 0 0788 gm. of CO_2 and 0 0206 gm of H_2O

0 1856 gm of the substance employed for Kjeldahl nitrogen estimation required for neutralization 6 90 cc of 0 1 n acid

0 2782 gm of the substance gave 0 0573 gm. of $Mg_2P_2O_7$

	Calculated for $C_9H_{11}N_3PO_9Pb$ per cent	Found per cent
C	20 40	20 08
H	2 10	2 21
N	5 29	5 21
P	5 86	5 74

Brucine Salt of Uridinphosphoric Acid.—About 4 0 gm. of the neutral ammonium salt were converted into the lead salt. This was freed from lead by means of hydrogen sulfide and the brucine salt was obtained in the usual way. The salt was recrystallized twice, each time out of 1,500 cc. of 35 per cent alcohol.

The air-dry substance effervesced without becoming transparent at 185°C. (corrected) and contracted and melted without further decomposition at 195°C. (corrected)

The air-dry substance had the following composition.

0 0996 gm of the substance gave 0 1948 gm of CO_2 and 0 0591 gm of H_2O

0 2000 gm of the substance gave 12 8 cc of nitrogen gas at $T° = 24°C$, at $P = 759$ mm

0 3000 gm of the substance gave 0 0270 gm of $Mg_2P_2O_7$

	Calculated for $C_9H_{13}N_2PO_9(C_{23}H_{26}N_2O_4)_2+7H_2O.$ per cent	Found per cent
C	53 30	53 33
H	6 43	6 64
N	6 79	7 35
P	2 50	2 51

The rotation of the substance, owing to the insolubility of the substance, was taken in great dilution, and was as follows.

$$[\alpha]_D^{20} = \frac{-0\ 16 \times 100}{2 \times 0\ 4} = -20\ 0$$

ON THE IDENTITY OF THE WATER-SOLUBLE GROWTH-PROMOTING VITAMINE AND THE ANTINEURITIC VITAMINE.

By H. H MITCHELL.

(From the Department of Animal Husbandry, University of Illinois, Urbana.)

The vitamine requirements of animals have been investigated along two distinct l nes; *i.e.*,(1) through a study of the nutritive deficiencies of rations containing only known chemical compounds, and (2) through a study of the prevention and cure of certain diseases definitely referable to faulty diet   By the first line of investigation it has been repeatedly demonstrated that, besides the well known constituents of plant and animal tissues, the higher mammals require for the maintenance of health, and even of life, and for the production of normal growth small amounts of substances of unknown composition, more or less widely distributed throughout natural food products.   At least two such substances are required.   One is associated with certain animal fats and fresh leafy vegetables especially, and various y referred to as the fat-soluble vitamine or growth-promoting substance, or as fat-soluble A, a convenient abbreviated term introduced by McCollum and Kennedy, which unfortunately has not the general currency that it deserves.   The other growth-promoting substance is never associated with fats, but is especially rich in the g andular tissues of animals, hens' eggs, the embryos of seeds, and the leaves of plants   It is spoken of as the water-soluble growth-promoting vitamine or as water-soluble B (McCollum). Any assertion that the common sources of either of these vitamines contain only one ind.spensable substance is based purely upon circumstantial evidence

By the second 'ine of investigation it has been shown with reasonable certainty that there are at least two diseases referable to faulty diet, the cause of which, in each case, is a deficiency in

the diet of an unknown substance associated with natural food products.   These two diseases, beri-beri and scurvy in man (or polyneuritis in pigeons and scurvy in guinea pigs), are "deficiency diseases" in the sense that they may result when an animal subsists on food containing all the known and well recognized constituents of a complete diet, and may be cured, except for serious organic lesions, by adding to such a diet small amounts of extracts of certain natural food products.   The unknown substances involved in the production of these two deficiency diseases have been called the antineuritic vitamine and the antiscorbutic vitamine, respectively, and their distribution in plant and animal tissues, their solubilities, and chemical properties have been the subject of a large amount of research. Besides these two deficiency diseases, no others are generally recognized as such.   A condition of sore, inflamed eyes, which, according to McCollum, should be diagnosed as xerophthalmia, often results from a deficiency of sources of fat-soluble A in the diet.   While McCollum definitely classifies this condition as a deficiency disease, it does not seem to be generally recognized as such, and a reasonable doubt may exist as to whether it invariably results from a deficiency of fat-soluble A and whether its etiology does not involve an infection avoidable by exercising proper sanitary measures alone.[1]

It is evident that the numerous recent investigations on vitamines that have been undertaken by many workers, especially in America and England, have seriously complicated the problem of the nutritive requirements of animals.   Any experimental evidence that would simplify this situation would therefore be doubly welcome.   However, an undue simplification, based upon insufficient evidence, would lead to confusion and might seriously impede progress.   One possibility that would simplify matters would be a clear-cut demonstration that two or more of these vitamines are in fact identical   That fat-soluble A and water-soluble B are identical, or that the antineuritic and antiscorbutic vitamines are identical, may be ruled out at once on the evidence at present available.   However, the identity of water-soluble B and the antineuritic vitamine is a possibility not definitely dis-

[1] Bulley, E. C , *Biochem. J* , 1919, xiii, 103

posed of in this summary manner In fact, the belief exists in many quarters that such an identity exists. This belief ranges from positive conviction, through tacit acceptance, to a frank weighing of probabilities. On the one hand is the statement of McCollum and his coworkers that. "Xerophthalmia and poly-neuritis are abundantly demonstrated to have their origin in the lack of a sufficient amount of the fat-soluble A and water-soluble B respectively in the diet."[2] Evidently as the result of this unequivocal attitude of McCollum, some investigators use the terms "water-soluble B" and "antineuritic vitamine" inter-changeably Osborne and Mendel are more conservative: "Whether or not the antineuritic component [of yeast] is identical with the growth-promoting one is a question which as yet has received no definite answer . . . "[3] Again, after referring to studies of the protective, curative, or antineuritic properties of certain animal tissues, they say "That the substance which induces the remarkable recoveries which have been described in these cases is identical with the water-soluble hormone which is so essential for growth and maintenance is as yet merely a matter of conjecture."[4]

In view of the importance of the question of the identity of these two vitamines, one essential for the maintenance of life and growth, the other for the prevention of multiple neuritis, and in view of the lack of general acceptance of the affirmative statement of McCollum and coworkers, a somewhat searching critical consideration of the evidence may render a real service in clarifying the issue and indicating the most likely points of future attack.

The conclusion that the two vitamines are identical seems to be based upon the following grounds (1) The distribution of the two substances in natural food products is very similar and the correlation between the actual amounts (in as far as these have been measured by biological tests) found in different products appears to be close. (2) The lack of known sources of water-soluble B in the diet of various species of experimental animals

[2] McCollum, E V , Simmonds, N , and Parsons, H T , *J Biol Chem* 1918, xxxiii, 413. ·

[3] Osborne, T B , and Mendel, L B , *J Biol Chem* , 1917, xxxi, 154.

[4] Osborne, T B , and Mendel, L B , *J Biol Chem* , 1917, xxxii, 311.

seems generally, if not always, to result in symptoms of nerve degeneration and paralysis. (3) Extracts of natural food products possessing growth-promoting properties are said to contain very probably only one indispensable vitamine, though supplementing satisfactorily a ration containing no other possible source of antineuritic vitamine. (4) The solubilities of the two vitamines in the common solvents are said to be identical   (5) Attempts at isolating the two vitamines have shown that they possess identical precipitants and adsorbents.   (6) The stabilities of the two substances, especially to acids, alkalies, and elevated temperatures, seem to be similar if not identical

Critical analyses of these points are presented in order.

1. While the distribution of the two vitamines among natural food products is strikingly similar in many respects, there are some instances where the correlation is not close   Through the work of Osborne and Mendel,[5] it has been shown that green vegetables, such as cabbage and spinach, are rather rich sources of water-soluble B, compared, for example, with the whole cereal grains.   McCollum and Kennedy,[6] however, have found that cabbage contains the antineuritic factor in "a quite low concentration," a conclusion confirmed by some recent work of Chick and Hume.[7] These investigators have concluded from experiments on the relative vitamine content of a large range of different foodstuffs that fresh and desiccated vegetables, including the cabbage, onion, and carrot, are poor sources of the antineuritic vitamine as compared with whole wheat, being about on a par with fresh meat   Fresh meat is known to be a poor source of both water-soluble B and the antineuritic vitamine,[8] but, on the other hand, the carrot seems to be rich in water-soluble B,[9] though poor in antineuritic vitamine.   Again, Chick and Hume have found the potato to have a very low content of antineuritic vitamine, having practically no value as a preventive against avian

[5] Osborne, T B , and Mendel, L B , *J Biol Chem* , 1919, xxxvii, 187

[6] McCollum, E V , and Kennedy, C , *J Biol Chem* , 1916, xxiv, 496

[7] Chick, H , and Hume, E M , *Tr Soc Trop Med and Hyg* , 1916–17, x, 156

[8] Osborne, T B , and Mendel, L B , *J Biol Chem* , 1917, xxxii, 309
Cooper, E A , *J Hyg* , 1912, xii, 436, 1914, xiv, 12

[9] Sugiura, K , and Benedict, S R , *J Biol Chem* , 1918, xxxvi, 171, 191.

polyneuritis, a result confirmed by Vedder and Clark,[10] though McCollum and Kennedy claim that potato juice possesses a moderate curative power   As regards its content of water-soluble B, however, McCollum, Simmonds, and Parsons[11] have shown that the potato, when constituting 84 5 per cent of the ration of rats, provides enough of this vitamine for normal growth. Chamberlain, Vedder, and Williams[12] found water extract of onions to have no curative effect on polyneuritic pigeons, though Osborne and Mendel[13] discovered considerable water-soluble B in this food as well as in turnips, beets, and tomatoes.

The green vegetables, and roots and tubers seem in general, therefore, to contain rich or moderate amounts of the growth-promoting water-soluble vitamine, and small amounts or none at all of the antineuritic vitamine.   If this relation is abundantly confirmed by future research, it may of itself effectively dispose of any contention of the identity of the two vitamines   The evidence as it stands, however, does not amount to finality for several reasons.   Much recent work has shown that the vitamine content of fresh vegetables, and roots and tubers is extremely variable, depending apparently on the freshness and maturity of the material.   No experiments appear to have been carried out on the antineuritic and the growth-promoting properties of the same vegetable samples.   Again, the experimental polyneuritis of pigeons is almost invariably induced by a diet of polished rice in investigations on the comparative antineuritic properties of foods.   Polished rice, however, is defective in several factors besides the antineuritic factor.   It is therefore probable that foods containing comparable amounts of this vitamine may be unequally effective in preventing or curing the polyneuritic symptoms, depending upon the extent to which they supplement polished rice in these other respects.   Conversely, foods containing unequal concentrations of the antineuritic principle may have their relative values as sources of the vitamine distorted for

[10] Vedder, E B , and Clark, E , *Philippine J Sc ,Section B*,1912,vii, 423

[11] McCollum, E V., Simmonds, N , and Parsons, II T , *J Biol Chem ,* 1918, xxxvi, 208

[12] Chamberlain, W P , Vedder, E B , and Williams, R R , *Philippine J Sc , Section B*, 1912, vii, 45.

[13] Osborne, T B , and Mendel, L B , *J Biol Chem* , 1919, xxxix, 29

the same reason.   The argument is illustrated by the experience
of Campbell and Chick with scorbutic rations [14]   A ration of
oats, bran, and water produces death from scurvy in guinea pigs
in 3 to 4 weeks.   However, the addition of 60 cc of autoclaved
milk, a food containing no demonstrable antiscorbutic value,
delays the onset of scurvy from 1 to 3 weeks, evidently simply
by correcting the deficiencies of the basal ration in factors other
than the antiscorbutic.

Further evidence, out of harmony with the assumption that
water-soluble B and the antineuritic vitamine are identical, is
afforded by experiments on unpolished rice   McCollum and
Davis [15] have shown reason to believe that unhusked rice is ade-
quately supplemented by casein, salts, and butter fat, similar to
the other cereal seeds   Gibson and Concepcion, [16] however, report
experiments on pigeons indicating an incomplete protection against
polyneuritis afforded by an exclusive diet of unhusked rice.   Six
fowls were fed on palay (unhusked rice), three for 2 months,
two for 3 months, and one for 4 months.   While they de-
veloped no symptoms of neuritis in this time, on postmortem
examination, the sciatic nerves showed distinct degenerative
changes in every case on being stained by the Marchi method.
The degeneration corresponded to that obtained with birds fed
for 2 weeks or more on milled rice.   In fact, the degeneration
in two of the birds was more pronounced than can be observed
in some subjects that have died of rice polyneuritis.   In the case
of man, also, analogous results have been reported. [17]

2. The lack of known sources of water-soluble B in the diet of
rats has frequently been said to result in symptoms of paralysis
of the hind legs, and the conclusion has been drawn that a well
defined neuritis existed.   Such symptoms, however, are not uni-
versally noted as a result of subsistence on rations totally lacking
in water-soluble B, [18] and the conclusion that they invariably

[14] Campbell, M E D   and Chick, H , *Lancet*, 1919, ii, 320

[15] McCollum, E V , and Davis, M , *J Biol Chem* , 1915, xxiii, 230

[16] Gibson, R B , and Concepcion, I , *Philippine J Sc* , *Section B*, 1914,
ix, 119

[17] Strong, R P , and Crowell, B C , *Philippine J Sc* , *Section B*, 1912,
vii, 414   Shibayama, *J Trop Med and Hyg* , 1913, xvi, 284

[18] Osborne, T B , Wakeman, A J , and Ferry, E L , *J. Biol Chem* ,
1919, xxxix, 35

develop cannot be verified   Even when animals do develop such symptoms it does not seem to be specific to a deficiency of this vitamine.   Hart, Miller, and McCollum[19] report experiments on pigs on a large number of rations, leading in many cases to paralysis of the hind quarters, and, as brought out by the histological examination of sections of the spinal cord, to marked edema and degeneration of the motor cells.   They conclude from this work.

"Malnutrition, histologically characterized by nerve degeneration, may result from the absence of certain factors in the diet as in the case of beri-beri   A similar condition may likewise arise from the presence of toxic materials in apparently normal food products, and in the presence of all known factors essential for continued growth and well-being "

It can hardly be contended, therefore, that the appearance of neuritic conditions in experimental animals is an unequivocal indication of a deficiency in antineuritic vitamine

3.   The existence of two indispensable unknown substances in water and alcohol extracts of natural foods is doubted by McCollum and Simmonds[20] on the basis of the following evidence.

"In the experimental part of this paper it is shown that the water-soluble B is not extracted directly from beans, wheat germ, or pig kidney by ether, benzene, or acetone, but is readily extracted in great part by alcohol.   After being removed by alcohol it is shown to be soluble in benzene, but very slightly soluble in acetone   The probability that there should be two or more physiologically indispensable substances in what we term water-soluble B, both or all of which should show the same solubility relations with three solvents, is relatively small and lends support to our view that the substance which protects animals against polyneuritis is the only essential complex in the extracts described."

While the argument is directed particularly against the theory that scurvy is a deficiency disease, it is just as forceful against any theory that water-soluble B and the antineuritic vitamine are not one and the same thing   Thus, McCollum's theory of the nutritive requirements of animals, which, because of its simplicity and the unequivocal terms in which it is stated, has

[19] Hart, E B , Miller, W S , and McCollum, E V , *J Biol Chem* , 1916, xxv, 239

[20] McCollum, E V , and Simmonds, N , *J Biol Chem* , 1918, xxxiii, 62.

gained many advocates, has no room for two vitamines possessing the solubilities of water-soluble B. The argument is not particularly impressive, being entirely of a circumstantial character. In fact, it is quite conceivable that the treatment of natural foods with boiling alcohol breaks up combinations of vitamines with other substances, so that removal of the water-soluble B by this solvent may have been a combination of chemical and purely solvent action. A crucial experiment that would dispose of this possibility would have been to boil the food with alcohol in a reflux condenser for a short time only, evaporate off the alcohol, and then test with acetone and benzene for extraction of the active substance.

4. The solubilites of the water-soluble B and the antineuritic vitamine are very largely similar. Both are soluble in water and dilute alcohol and are ordinarily stated to be insoluble in fat solvents. Solubility in absolute alcohol is variously stated for both substances. Osborne and Mendel, and Drummond[21] claim that the water-soluble growth-promoting substance of yeast is insoluble in absolute alcohol, and McCollum and Simmonds[20] have found it to be only incompletely soluble in 95 per cent alcohol. On the other hand, Eijkman[22] claims that the antineuritic substance of yeast is extracted by strong alcohol. The neuritic curative substance of rice polishings has been repeatedly extracted by Funk[23] and Fraser and Stanton[24] by absolute alcohol and appears to be readily soluble in this reagent. Cooper[8] has also shown that the antineuritic vitamine of dried beef is extracted by absolute alcohol, and that the constituent of egg yolk that cures polyneuritis in pigeons is readily extractable with ether[25] from the dried yolk. After extraction with ether, a further yield was obtained from the residue by means of absolute alcohol.

While the evidence for the solubility of the two vitamines in alcohol is incomplete and somewhat conflicting, though lending

[21] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 158. Drummond, J. C., *Biochem. J.*, 1917, xi, 261.

[22] Eijkman, C., *Arch. Schiffs- u. Tropenhyg.*, 1911, xv, 698.

[23] Funk, C., *J. Physiol.*, 1911–12, xliii, 395.

[24] Fraser, H., and Stanton, A. T., *Lancet*, 1910, ii, 1755.

[25] McCollum and his coworkers have repeatedly shown that water-soluble B is not extracted by ether from food materials.

suppoit to the conclusion that the growth-promoting factor is considerably less soluble than the antineuritic, some clear-cut evidence exists of a distinct difference in solubility in acetone and benzene, mainly from the work of McCollum and his associates. Thus, McCollum and Kennedy[26] conclude from many experiments on the curative properties of various extracts of wheat embryo for polyneuritic pigeons that acetone and benzene extract from this material, previously rendered fat-free by extiaction with ether, the substance which relieves the symptoms of polyneuritis in pigeons. Alcohol (95 per cent), water, acetone, and benzene extracts of fat-free wheat embryo were all shown to be capable of curing polyneuritic pigeons, and, as far as can be judged from the protocols of the experiments, the cuie was just about as readily accomplished with acetone and benzene extracts as with alcohol and water extracts. At most, the superiority of the latter was slight

Entirely different results were obtained by McCollum and Simmonds[27] in later investigations on the solubility of water-soluble B. In investigating the growth-promoting properties of extracts of raw and cooked navy beans, wheat embryo, and pig kidney added to a basal ration deficient only in water-soluble B, they were able to show that acetone and benzene do not extract the water-soluble B from these products to any appreciable extent. Slight indications that the acetone extract carried traces of this vitamine were encountered, but they were still led to believe that most of the substance remained in the residue. This work confirmed some previous work by McCollum and Davis[28] on the supplementary action of acetone extracts of wheat embryo on polished rice After extraction of the water-soluble B by alcohol, it was found by McCollum and Simmonds to be only slightly soluble in acetone, and many times more soluble in benzene

[26] McCollum, E V , and Kennedy, C , J Biol Chem , 1916, xxiv, 491.
[27] McCollum, E V , and Simmonds, N , J Biol. Chem , 1918, xxxiii, 55
[28] McCollum, E V , and Davis, M , J Biol Chem , 1915, xxiii, 229, 230
This investigation and the pieceding one aie open to the ciiticism that no food intake iecords aie given in ordei that the ieadei may judge whether a change in iation was effective by reason of a difference in food intake oi of a difference in food composition, and also that the expeiimental periods were often too shoit to indicate any effect at all

Recently Steenbock[29] has reported that by means of neutral solvents there was prepared a water-acetone-soluble fraction from egg yolk which in small doses by intraperitoneal injections was able to cure a pigeon suffering from polyneuritis.

From the work quoted, therefore, the evidence lends support to the conclusion that the water-soluble B is not extracted from food materials to any appreciable extent by either acetone or benzene, and that when extracted it is still only slightly soluble in acetone, though readily soluble in benzene.   The antineuritic vitamine, however, seems to be readily extracted from wheat embryo by acetone and benzene, and to be readily soluble in both of these solvents.

5. Attempts to isolate the antineuritic vitamine from rice polishings and yeast have been much more numerous and have given more definite results than similar attempts with the water-soluble B.   The antineuritic vitamine has been shown to be quantitatively precipitated by phosphotungstic acid in 5 per cent sulfuric acid solution, giving a phosphotungstate insoluble in acetone   It is also completely precipitated by silver nitrate and barium hydroxide, only partially precipitated by mercuric chloride, and is not precipitated by platinic chloride.   It is quantitatively adsorbed by animal charcoal, fullers' earth, and by hydrated aluminium silicate (Lloyd's reagent).   The antineuritic vitamine is also dialyzable

Funk and Macallum[30] have attempted a similar fractionation of the water-soluble B from yeast with indifferent success   They showed that phosphotungstic acid precipitated the growth-promoting substance, and that the filtrate from this precipitation was inactive.   However, only a small fraction of the growth-promoting capacity of the yeast was found to have survived this precipitation.   Subsequent decomposition of the precipitate and reprecipitation with silver nitrate and baryta precipitated a fraction, whose growth-promoting effect was "not sufficiently marked to encourage further investigation."   Lloyd's reagent was also used as a precipitant without much success, as the rats on the filtrate also showed increments in growth.   According to the authors:

[29] Steenbock, H , *J Biol Chem* , 1917, xxix, p xxvii
[30] Funk, C , and Macallum, A B , *J Biol Chem* , 1916, xxvii, 63

"The results obtained so far clearly indicate that the growth-promoting substance is analogous to and possibly identical with the beri-beri vitamine . . . However, it must be admitted that while it is uncertain whether these two substances are chemically different, the results obtained do not exclude such a possibility"

Eddy[31] showed that the water-soluble portion of the alcoholic extract of sheep pancreas contains a substance capable of inducing marked increase in growth when added to a ration very low, if not lacking, in vitamines. This substance was removed from the extract with Lloyd's reagent, though the completeness of removal was not tested. It was also precipitated by phosphotungstic acid. A criticism of the work of Funk and Macallum and of Eddy is that the basal ration used in making the biological tests, besides their deficiency of water-soluble B, could not have contained more than a trace of fat-soluble A

A more extensive and better planned experiment was reported by Drummond,[32] investigating the water-soluble B from yeast. Drummond was able to show that the growth-promoting substance is dialyzable. From the dialysate phosphotungstic acid precipitated a fraction possessing very slight growth-promoting activity, while the unprecipitated fraction possessed none. The distinction between the two fractions was slight and in the absence of food intake records is not susceptible of unequivocal interpretation. In another experiment, better growth curves were obtained when the fraction represented by the phosphotungstates insoluble in acetone was used than when that contained in the phosphotungstates soluble in acetone was used. Even smaller differences were observed between the growth curves of these two groups, however, than in the preceding experiment. Upon fractionation of yeast dialysate with silver nitrate and baryta, the precipitate produced with silver nitrate (purine fraction) had no effect on growth, while that produced on the further addition of baryta (pyrimidine fraction) contained small traces of vitamine.

While Drummond's results support the view that water-soluble B and the antineuritic vitamine are identical, they do not constitute a demonstration. The lack of food intake records complicates their interpretation, while the large losses of vita-

[31] Eddy, W H , *J Biol Chem* , 1916, xxvii, 113

[32] Drummond, J C , *Biochem J* , 1917, xi, 255

mine as soon as precipitation was resorted to finds no parallel in analogous work with the antineuritic substance.   For example, Funk showed that the water solution of an alcoholic extract of rice polishings when tested on pigeons suffering from polyneuritis was effective in doses corresponding to about 20 gm. of the original polishings.   On precipitation of the solution with phosphotungstic acid, decomposition of the precipitate with baryta, precipitation of the excess barium with sulfuric acid, neutralization and evaporation *in vacuo*, and extraction of the residue with absolute alcohol, the extract was found to be effective as a curative for polyneuritic pigeons in doses corresponding to about 40 gm. of the original polishings   This would indicate a loss of some 50 per cent in antineuritic efficiency, a much smaller loss than seems to result with water-soluble B, even when the manipulations after the phosphotungstic acid precipitation are simplified by decomposing the precipitate with an amyl-alcohol-ether mixture, and eliminating the absolute alcohol extraction.   The ready adsorption of water-soluble B by precipitates of all descriptions postulated by Drummond, in explaining the large losses during chemical manipulation, does not seem to be a property of the antineuritic vitamine.   Thus, Emmett and McKim[33] show that while this vitamine is adsorbed by fullers' earth and Lloyd's reagent it is not adsorbed by the kieselguhrs or infusorial earths, indicating a selective adsorption by the former.   Being readily dialyzable, there seems to be no compelling reason for believing it to be indiscriminately adsorbed by precipitates of all kinds.

6. The stability of both water-soluble B and the antineuritic vitamine to acids seems to be great   Even boiling the vitamines with concentrated mineral acids does not seem to destroy them to any appreciable extent, though some results reported by Drummond[32] may be interpreted as indicating a partial destruction of water-soluble B by boiling with 20 per cent sulfuric acid for 10 hours, a result not in harmony with the work of Funk on antineuritic vitamine.   Both vitamines seem to be stable to even concentrated alkalies at room temperature.[34]   At the boiling tempera-

[33] Emmett, A D , and McKim, L H , *J Biol Chem* , 1917, xxxii. 409
[34] The conclusion of Fraser and Stanton (Fraser, H , and Stanton, A T , *Lancet*, 1915, i, 1021), to the effect that exposure of antineuritic preparations to 0 5 per cent sodium hydroxide at room temperature very quickly destroyed their curative properties for polyneuritic pigeons, has not been confirmed by recent investigations

ture the antineuritic vitamine seems to be very rapidly destroyed by alkalies,[29] though experiments on this point are too few to warrant drawing definite conclusions. Water-soluble B does not seem to be destroyed particularly rapidly by dilute alkalies at high temperatures. While Drummond[32] found that hot 5 per cent sodium hydroxide in 5 hours tends to destroy the growth-promoting properties of yeast preparations, Osborne, Wakeman, and Ferry[18] were unable to detect the slightest destruction of this vitamine in dry brewers' yeast after digesting for 21.5 hours with 0.1 N sodium hydroxide and subsequently heating on the water bath for 2 hours. The conflicting results of McCollum and Simmonds,[20] and of Daniels and McClurg[35] on alkali of greater strength cannot at present be evaluated. For the proper solution of the question, food intake records must be considered.

While both water-soluble B and antineuritic vitamine do not seem to be destroyed by long exposure to a temperature of 100°C. or even slightly higher, the evidence is conflicting for temperatures of 120°C. Early investigations on the antineuritic vitamine in general substantiate the conclusion that 1 to 2 hours exposure to this temperature either totally destroys the antineuritic efficiency or markedly lowers it The recent systematic investigation of Chick and Hume[36] indicates that the curative properties of wheat embryo and yeast extract are rapidly destroyed at 120°C. Wheat embryo, heated at 118–124°C. for 2 hours, failed to effect a complete cure of polyneuritis in pigeons when given in doses four times as large as the effective dose of unheated embryo. Heated at 110–117°C. for 40 minutes, it required more than twice as much embryo Experiments with yeast extract showed a slower destruction of the antineuritic substance, since when heated at 120°C. for 2 hours 10 cc of extract seemed to be as effective in curative action as 4 cc. of the unheated extract

Unfortunately no strictly quantitative work on the effect of heat on water-soluble B, comparable to the investigations of Chick and Hume on the antineuritic vitamine. has been reported. McCollum and Davis[37] have shown that rats grow normally on

[35] Daniels, A L , and McClurg, N I , *J Biol Chem* , 1919, xxxvii, 201

[36] Chick, H , and Hume, E M , *Proc Roy Soc London, Series B*, 1917–19, xc, 60

[37] McCollum, E V , and Davis, M , *J Biol Chem* , 1915, xxiii, 247

rations containing 10 per cent of evaporated whey, heated in an autoclave at 15 pounds pressure (120°C.) for 1 hour, as the sole source of water-soluble B. In view of the low content of milk in this vitamine it does not seem probable that any appreciable destruction could have occurred in this experiment. In another experiment, 13.3 per cent of wheat embryo heated in the autoclave for 1 hour served as the sole source of water-soluble B, supporting normal growth and reproduction McCollum, Simmonds, and Pitz[38] showed that rations containing as low as 25 per cent of navy beans, moistened, and heated in the autoclave for 75 minutes at 15 pounds pressure, supported normal growth and reproduction in rats Daniels and McC urg[35] heated navy beans, soy beans, and cabbage in a pressure cooker for 15 to 40 minutes at 120°C and found them to be suitable sources of water-soluble B in the ration of rats when included in excessive amounts. No definite conclusions can be deduced from this experiment, however, as to the rate of destruction of the vitamine if any destruction occurred. Drummond[32] found that heating yeast dialysate for 30 minutes at 120°C impaired its value as a source of water-soluble B, but the growth curves illustrating his experiment afford no basis for assuming any great destruction of vitamine, since only 6 per cent of the preparations tested was included in the rations as the sole source of water-soluble B.

The experiments just reviewed on the stabilities of the water-soluble growth-promoting vitamine and of the antineuritic vitamine, afford no sure basis of distinction though they are suggestive as indicating a greater stability of the former to hot alkali and to temperatures above 100°C.

In evaluating the data on the occurrence and properties of the two vitamines cited above, there seems to be very good reason for doubting their identity. In settling the question definitely, however, there is need of experiments in which different foods and preparations from foods are tested both for their growth-promoting properties and their curative effects on polyneuritic pigeons. Until such work is done in a quantitative way, dogmatic assertion that a lack of water-soluble B leads to polyneuritis or beri-beri, or an interchangeable use of the terms

[38] McCollum, E. V , Simmonds, N , and Pitz, W , *J Biol Chem* , 1917, xxix, 521.

"water-soluble B" and "antineuritic vitamine" serves no purpose, tending rather to impede progress in this direction. It is significant that while many investigators in nutrition regard the etiology of beri-beri as definitely cleared up, the medical profession in those parts of the world where beri-beri is endemic are not at all convinced of the fact, and still believe that a definite infection is involved along with malnutrition.[39] Furthermore, Gibson and Concepcion[16] say that in the Philippine Islands the therapeutic use of rice bran or extracts and preparations of rice bran has not given the specific results for man which were expected from theoretical considerations of the etiology of the disease, although the mortality has been greatly reduced.   Williams and Johnston[40] also are veering away from the orthodox view of the cause of beri-beri in favor of a toxic view.   They have been able to transmit polyneuritis to a pigeon, subsisting on unhusked rice, by feeding it the minced internal organs of birds that had died of the disease.   This bird, 9 days after the ingestion of the diseased organs, developed all the typical symptoms of polyneuritis precisely as do birds fed on polished rice.   The 2nd day thereafter the bird became completely prostrated, displayed increasingly severe retraction of the neck and labored breathing, and died.   In view of such reports it cannot be doubted that many points in the etiology of polyneuritis and of beri-beri still need to be cleared up, and that premature deductions from a limited amount of experimental data do not hasten but can only defer the ultimate solution.

[39] See for example the discussion by medical men of the paper of Chick and Hume presented before the Society of Tropical Medicine and Hygiene (Chick, H , and Hume, E M , Tr Soc Trop. Med and Hyg , 1916–17, x, 179–186)

[40] Williams, R R , and Johnston, J A., Philippine J Sc , Section B, 1915, x, 337

THE STRUCTURE OF YEAST NUCLEIC ACID.

IV. AMMONIA HYDROLYSIS.

By P A LEVENE.

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The tetranucleotide theory of the structure of yeast nucleic acid was first enunciated by the writer[1] and was subsequently conclusively demonstrated by the experimental evidence furnished by Levene and Jacobs,[2] and by Levene and La Forge.[3] The facts on which the theory was based were first, the isolation of four nucleosides, second, the isolation of simple pyrimidine nucleotides These were obtained on partial hydrolysis of yeast nucleic acid The third fact, important for the development of the theory, was the elucidation of the order of linkage of the components of one simple mononucleotide [4]

The experimental data obtained until that phase of work permitted no rational formulation of the mode of linkage between individual mononucleotides Our original graphic representation of the entire molecule of yeast nucleic acid had only an arbitrary schematic sense In a publication on thymus nucleic acid, Levene and Jacobs made that point clear. Owing to pressure of other work, our own investigations into the problem of the linkage of the mononucleotides was making slow progress, when Thannhauser with his collaborators,[5] and Jones with his collaborators[6] entered the field of nucleic acid study

[1] Levene, P A , *Biochem Z* , 1909, xvii, 121 ·

[2] Levene, P A , and Jacobs, W A , *Ber. chem Ges* , 1909, xlii, 2475, 2703, 1910, xliii, 3151, 1911, xliv, 1027

[3] Levene, P A , and La Forge, F B , *Ber chem Ges* , 1912, xlv, 608, 3164

[4] Levene, P A , and Jacobs, W A , *Ber chem Ges* , 1908, xli, 2704; 1909, xlii, 335, 1198

[5] Thannhauser, S J , *Z physiol Chem* , 1914, xci, 329   Thannhauser, S J , and Dorfmuller, G , *ibid* , 1917, c, 121

[6] Jones, W , and Richards, A E , *J Biol. Chem* , 1914, xvii, 71.   Jones, W , and Germann, H C , *ibid* , 1916, xxv, 93   Jones, W , and Read, B E , *ibid* , 1917, xxix, 123; xxxi, 39.

By means of either enzyme action or by methods of chemical hydrolysis, they obtained intermediate substances which were regarded by them as di- and trinucleotides.

The methods of hydrolysis employed by them in some instances were identical with those employed by us in other instances, slightly modified   The method of separating simple nucleotides as their brucine salts was also introduced by us.

Thannhauser first announced the isolation of a trinucleotide from the products of digestion of nucleic acid by enzymes Later, Thannhauser and Dorfmuller hydrolyzed nucleic acid by means of 25 per cent ammonia and supposedly cleaved the molecule into uridinphosphoric acid and a trinucleotide containing the remaining three nucleotides.   On acid hydrolysis with 2 per cent sulfuric acid these authors obtained only uridinphosphoric acid. This publication appeared in Germany in 1917 and did not reach us until 1919.   In 1914, Jones and Richards described experiments by which they thought they had cleaved nucleic acid into two dinucleotides, guanin-cytosine, and adenin-uridin .dinucleotides.   Subsequently, Jones and his coworkers described the same two dinucleotides which they obtained on hydrolysis of yeast nucleic acid by heating the acid in an autoclave in a 2 5 per cent ammoniacal solution for 1½ hours at a temperature of 115°C.   On the other hand, on hydrolysis by means of dilute acids, Jones and Read described a cytidin-uracil dinucleotide. On the basis of their respective findings, Jones and Thannhauser presented theories of the mode of linkage between the nucleotides.

According to Jones the structure of yeast nucleic acid is as follows.

$$\begin{array}{l}
\mathrm{OH}\diagdown \\
\quad \mathrm{O=P-O\ C_5H_7O_2\cdot C_5H_4N_5} \\
\mathrm{OH}\diagup \qquad | \\
\qquad\qquad\quad \mathrm{O} \\
\mathrm{OH}\diagdown \qquad | \\
\quad \mathrm{O=P-O\ C_5H_9O\cdot C_4H_3N_2O_2} \\
\mathrm{OH}\diagup \qquad | \\
\qquad\qquad\quad \mathrm{O} \\
\mathrm{OH}\diagdown \qquad | \\
\quad \mathrm{O=P-O\ C_5H_6O\cdot C_4H_4N_3O} \\
\mathrm{OH}\diagup \qquad | \\
\qquad\qquad\quad \mathrm{O} \\
\mathrm{OH}\diagdown \qquad | \\
\quad \mathrm{O=P-O.C_5H_7O_2\cdot C_5H_4N_5O} \\
\mathrm{OH}\diagup
\end{array}$$
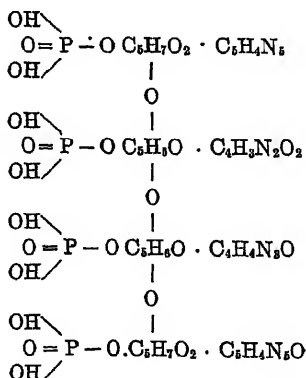
Thannhauser, on the other hand, presents the linkage of the nucleotides in the following way.[7]
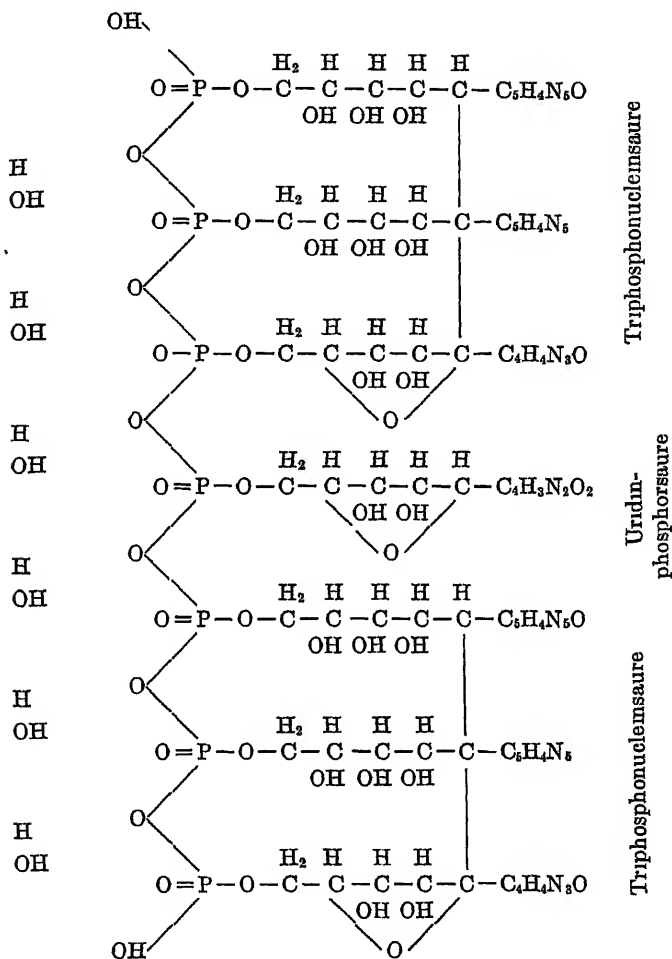
[7] In Thannhauser's representation there is an oversight in regard to the cytidin linking.

It is given   It should be

$$R - N - CNH_2 \qquad\qquad R - N - CH$$
$$\quad\ |\quad\ | \qquad\qquad\qquad\quad\ |\quad\ \|$$
$$\quad\ CO\quad CH \qquad\qquad\qquad CO\quad CH$$
$$\quad\ |\quad\ \| \qquad\qquad\qquad\quad\ |\quad\ |$$
$$\ HN - CH \qquad\qquad\qquad N = CNH_2$$
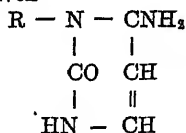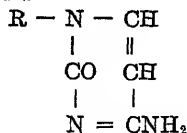
These theories are based on the following considerations:

That of Jones on the assumption of the reality of the dinucleotides, and on the observations that the so called dinucleotides are tetra-basic.   The theory of Thannhauser is based on the assumption of the belief in the reality of the trinucleotide, and second, on the fact that the so called trinucleotide is hexo-basic.

In previous communications we have criticized the conclusions of these writers, on the assumption that their observations were correct[8]   In a later communication,[9] we have shown that the cytidin-uridin dinucleotide was a mixture of uridin and cytidin mononucleotides.   Uridinphosphoric acid was obtained as a crystalline barium salt.   It may be mentioned here that optical rotation of the crystalline salt air-dry was $[\alpha]_D^{20} = 3\ 5$, or dry and barium-free $[\alpha]_D^{20} = 5\ 83$, whereas Thannhauser and Dorfmuller found for their uridinphosphoric acid $+ 14.4$.

On the other hand the barium salt of the cytidinphosphoric acid had the optical rotation of $[\alpha]_D^{20} = + 14\ 0$ or barium-free and dry $[\alpha]_D^{20} = + 23.3$ which agrees with the recent finding of Thannhauser for the crystalline cytidinphosphoric acid, which was $[\alpha]_D^{20} = 23$ Thus it is possible that the substance described by Thannhauser as uridinphosphoric acid was of a lesser degree of purity than that of the cytidinphosphoric acid.

In a still later publication[10] we reported on the finding that the so called cytidin-uridin dinucleotide was fractionated by us into uridinphosphoric and adenosinphosphoric acids.   The former was identified as the crystalline barium salt, the latter as the brucine salt, which at the time of that publication was converted into the barium salt   Since from a large quantity of brucine salt there was obtained only a small quantity of a barium salt analyzing satisfactorily for the salt of the adenosin nucleotide, the publication of the analytical data on that nucleotide was delayed   It was subsequently found that adenosinphosphoric acid is identified most conveniently as the free acid.   Crystalline adenosinphosphoric acid was described by Jones and Kennedy,[11] the substance obtained by us differed from that of

[8] Levene, P A., *J Biol Chem*, 1917, xxxi, 591

[9] Levene, P A., *Proc Soc Exp Biol and Med*, 1917, xv, 21

[10] Levene, P A, *J Biol Chem*, 1918, xxxiii, 425

[11] Jones, W., and Kennedy, R P, *J Pharmacol and Exp Therap*, 1919, xiii, 45

Jones only in the fact that our material dried in air contained no crystal water, whereas the substance of Jones contained one crystal water. The optical rotation of our substance is $[\alpha]_D^{20} = -38.5$ Whereas the rotation of adenosinphosphoric acid remains constant either in water or in 5 per cent ammonia water, the rotation of guanosinphosphoric acid shows a marked increase in its levorotation in ammonia water This point may serve for differentiation between the two nucleotides. Thus it is proven that the adenin-uridin dinucleotide is a mixture of two mononucleotides.

The fraction which was originally regarded by Jones as a guanin-cytosine dinucleotide, and from which Read isolated an amorphous guanylic acid, was also found by us to consist principally of guanosinphosphoric acid In addition a small proportion of uridinphosphoric acid was found in this fraction. The presence of the latter nucleotide might have escaped isolation if not for its recently described property of forming a crystalline lead salt

The guanylic acid was isolated in a crystalline form, and constituted the greater part of the fraction

The method of hydrolysis employed by us consisted in heating the nucleic acid in a 2.5 per cent aqueous ammonia solution for 1 hour at 100°C Thus the treatment was milder than the one employed by either Jones or Thannhauser Thus the present findings nullify the experimental evidence in support of the theories of Jones and of Thannhauser. From the theoretical point of view, the theory of Thannhauser is not very tenable for the reason that a carbon to carbon-linking implies a very strong union, whereas the polynucleotide is readily dismembered into mononucleotides Thannhauser, in fact, accepts it himself with great reserve. As regards the ether-linking accepted by Jones, it must be remarked that, as a rule, an ether-linking represents a very firm union If one accepts that this rule does not apply to carbohydrates linked in ether form, he should present experimental evidence in support of this view

However, if the work of Jones and of Thannhauser failed to support their speculations regarding the mode of linkage of the mononucleotides, it has been of great importance in furnishing further proof of the nucleotide structure of yeast nucleic acid;

and also, in making it possible to show that the molecule of nucleic acid is readily decomposed into mononucleotides, and that the linkage between all nucleotides is of the same order

On the basis of considerations such as these the linkage of the nucleotides could be expressed most simply in the following way:

$$
\begin{array}{l}
\text{OH}\diagdown \\
\quad \text{O}=\text{P}-\text{O}-\text{C}_5\text{H}_7\text{O}_2 \quad \text{C}_5\text{H}_4\text{N}_5\text{O} \\
\text{OH}\diagup \qquad\qquad\qquad\quad | \\
\qquad\qquad\qquad\qquad\quad \text{O} \\
\qquad\qquad\qquad\qquad\quad | \\
\qquad\quad \text{O}=\text{P}-\text{O}-\text{C}_5\text{H}_7\text{O}_2 \cdot \text{C}_4\text{H}_4\text{N}_3\text{O} \\
\qquad \text{OH}\diagup \qquad\qquad\qquad\quad | \\
\qquad\qquad\qquad\qquad\qquad\quad \text{O} \\
\qquad\qquad\qquad\qquad\qquad\quad | \\
\qquad\qquad \text{O}=\text{P}-\text{O}-\text{C}_5\text{H}_7\text{O}_2 \cdot \text{C}_4\text{H}_3\text{N}_2\text{O}_2 \\
\qquad\quad \text{OH}\diagup \qquad\qquad\qquad\qquad | \\
\qquad\qquad\qquad\qquad\qquad\qquad\quad \text{O} \\
\qquad\qquad\qquad\qquad\qquad\qquad\quad | \\
\qquad\qquad\qquad \text{O}=\text{P}-\text{O}-\text{C}_5\text{H}_7\text{O}_2 \quad \text{C}_5\text{H}_4\text{N}_5 \\
\qquad\qquad\quad \text{OH}\diagup
\end{array}
$$
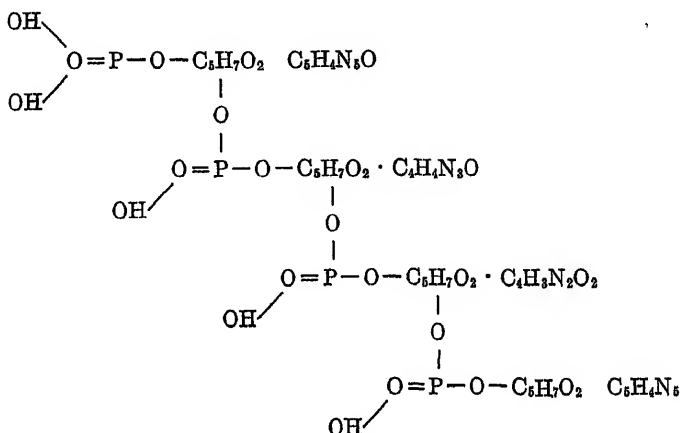
For the present this form expresses the facts known about the structure of yeast nucleic acid   New facts and new evidence may cause its alteration, but there is no doubt as to the polynucleotide structure of the yeast nucleic acid.

It is unfortunate that, owing to war conditions, the work of Thannhauser was not known to us earlier, also that apparently our work was not known to Thannhauser.

EXPERIMENTAL.

The mode of hydrolysis was practically the same as that described in a previous communication,[10] with a difference in one detail; namely, the temperature of the autoclave was maintained at 100°C.

Treatment of the product of hydrolysis was also the same as described in that communication, and essentially the same as employed by Jones and his collaborators. The fraction precipitated by 98 per cent alcohol will be referred to as guanin fraction, and that remaining in solution as adenin fraction.

Adenin fraction was treated in exactly the same manner as that described in the previous communication. The brucine salt was recrystallized nine times with boiling 35 per cent alcohol

The crystalline deposit consisted of uridinphosphoric acid previously described. The first three mother liquors, on concentration, gave a brucine salt containing C = 53 00, H = 6.40, and N = 10 per cent. The subsequent six mother liquors, on concentration, gave a brucine salt containing N = 8.5 per cent.

The brucine salt of the first three mother liquors was transferred into the ammonium salt   Originally the ammonium salts were converted into the barium salt   Barium salts, having analytical value sufficiently approaching that required by the theory, were obtained only after many purifications which were associated with much loss. Finally, an attempt was made to transform the ammonium salt into the free nucleotide. This was accomplished without difficulty in the following manner. To the hot solution of the ammonium salt, while the mixture was agitated, a hot solution of neutral lead acetate was added in a slow stream. When the necessary volume of lead acetate (25 per cent solution) was added, the mixture was brought to a boil and filtered   The precipitate was washed in a mortar and filtered; the operation was repeated three times. Finally the precipitate was suspended in water, treated with hydrogen sulfide, and the filtrate from lead sulfide was concentrated under diminished pressure at room temperature. On standing, adenosinphosphoric acid crystallized in long needles resembling the free nucleoside. The substance differed from that described by Jones in that it crystallized without crystal water. The analysis of the air-dry substance was as follows:

0 1010 gm of the substance gave 0 1268 gm of CO_2 and 0 0368 gm of H_2O

0.1000 gm of the substance employed for Kjeldahl nitrogen estimation required for neutralization 14.26 cc of 0 1 N acid

0 3000 gm of the substance gave 0 0940 gm of $Mg_2P_2O_7$

	Calculated for $C_{10}H_{14}N_6PO_7$. per cent	Found per cent
C	34 57	34 24
H	4 07	4 08
N	20 17	19.96
P	8 94	8 73

The optical rotation of the substance in aqueous solution was

$$[\alpha]_D^{20} = \frac{-0\,77 \times 100}{1 \times 2} = -38\,5$$

In a solution of 5 per cent ammonia water the rotation was

$$[\alpha]_D^{20} = \frac{-0\,80 \times 100}{1 \times 2} = -40\,0$$

Hydrolysis of the Adenosinphosphoric Acid.—2 gm. of the substance in 35 cc. of 1 per cent sulfuric acid were boiled over flame with reflux condenser for 1 hour. The product of hydrolysis was neutralized with sodium hydroxide and to the neutral solution aqueous picric acid was added as long as a precipitate formed The precipitate was dissolved in hot water and allowed to crystallize

The analysis of the air-dry substance was as follows

0 1000 gm of the substance gave 26 2 cc of nitrogen gas at $T° = 26°C$ and $P = 752$ mm

	Calculated for $C_5H_5N_5 \cdot C_6H_2(OH)(NO_2)_3 + H_2O$ per cent	Found per cent
N	29 32	29 60

The substance decomposed at 177°C (uncorrected).

Brucine Salt of Adenosinphosphoric Acid —2 gm. of the nucleotide were dissolved in hot water and the solution was neutralized with a solution of brucine in methyl alcohol. On cooling, the solution nearly solidified. The crystals of the brucine salt of the nucleotide were filtered off with suction, and the substance was recrystallized three times out of 35 per cent alcohol.

The air-dry substance on heating in a sealed capillary tube melted as follows At 177°C it began slightly to contract; at 195°, the substance effervesced, remaining perfectly colorless; at 225° a second point of effervescence was observed, the substance turning dark The substance analyzed as follows

0 1020 gm of the substance gave 0 1978 gm of CO_2 and 0 0572 gm of H_2O

0 2000 gm of the substance gave 17 6 cc of nitrogen gas at $T° = 24°C$, $P = 769$ mm

0 3000 gm of the substance gave 0 0244 gm of $Mg_2P_2O_7$

	Calculated for $C_{10}H_{14}N_5PO_7 (C_{23}H_{26}O_4N_2)_2 \cdot 7H_2O$ per cent	Found per cent
C	53 28	52 88
H	6 40	6 28
N	10 00	10 23
P .	2 47	2 27

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{-0\ 74 \times 100}{1 \times 2} = -37\ 0$$

The guanin fraction consisted principally of guanosinphosphoric acid (guanylic acid)   It was treated in a general way in the manner indicated by Read   The lead salts were converted into the brucine salts, and these were fractionated in the same manner as the salts of the adenin fraction   However, the substance obtained from all the nine mother liquors contained over 10 per cent of nitrogen and only the fraction constituting the ultimate crystalline deposit contained on analysis about 8 75 per cent of nitrogen.   Surprisingly also this fraction consisted in the main of guanylic acid.   The brucine salts were converted into the ammonium salts   These were dissolved in boiling water and to the hot solution a hot solution of lead acetate was added in a slow stream.   The mixture was then brought to a boil and filtered hot.   The lead precipitate was freed from lead and concentrated under diminished pressure at room temperature.   Generally an amorphous, somewhat gelatinous precipitate settles out.   In some instances the solution turns into a semiliquid jelly   To bring about final crystallization, no general rule can be given   At times repeated precipitation with lead acetate will lead to a filtrate which, on concentration, solidifies into a crystalline mass   Often it is advisable to precipitate the nucleotide by means of lead acetate fractionally.   The later fractions as a rule crystallize with less difficulty.

The properties and analysis of the crystalline guanylic acid were described in a previous communication.[12]

When the brucine salts with 8.75 per cent of nitrogen were converted into ammonium salts, and when these were taken up

[12] Levene, P A , *J Biol. Chem* , 1919, xl, 171

in hot water, part of the substance remained insoluble. This residue consisted of brucine salt which escaped, being converted into ammonium salt The brucine salt on analysis showed a nitrogen content of N = 7.8 per cent. This brucine salt was then converted into the ammonium salt. The latter was dissolved in boiling water, and a hot solution of neutral lead acetate was added. The mixture was brought to a boil and filtered hot. The filtrate was seeded with a few crystals of the lead salt of uridinphosphoric acid, and allowed to stand near a hot water bath. It was found that when the cooling of the filtrate proceeded rapidly a gelatinous lead salt settled out. If, however, the cooling was progressing slowly the lead salt of uridinphosphoric acid settled out in crystalline form. For analysis the substance was dried to constant weight under diminished pressure at the temperature of xylene vapor. It analyzed as follows

0.1118 gm of the substance gave 0 0856 gm. of CO_2 and 0.0218 gm of H_2O.

0 1848 gm of the substance employed for Kjeldahl nitrogen estimation required for neutralization 7.7 cc of 0.1 N acid

0 2772 gm of the substance gave 0 0574 gm of $Mg_2P_2O_7$

	Calculated for $C_9H_{11}N_2PO_9Pb$ per cent	Found per cent
C	20 40	20 73
H . .	2.10	2 18
N . .	5 29	5.84
P	5 86	5 78

THE ACTIVITY OF LUNG EXTRACT, AS COMPARED TO EXTRACTS OF OTHER TISSUES, IN INDUCING COAGULATION OF THE BLOOD.

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Wherry and Ervin (1), in experimenting with the intravenous injection of extracts of tuberculous lung tissue, found that very small doses of an extract of normal as well as tuberculous lung tissue would produce death in a few seconds in rabbits and guinea pigs. It was at their request that I undertook to discover the cause of the sudden death in these cases.

The extracts were made by grinding the fresh lung tissue well with sand in a mortar, adding gradually while stirring 10 cc. of 0.9 per cent NaCl for each gm of tissue taken. The mixture was then centrifuged for 20 to 30 minutes at about 3,000 revolutions per minute and the slightly cloudy, reddish solution used for the injections. It was found by trial that 0 3 cc of this solution injected *rapidly* into the ear vein of a rabbit weighing 1,000 to 1,500 gm. caused respiratory symptoms of irregular breathing and uneasiness in 20 to 30 seconds, weakness and prostration shortly afterwards, and death with convulsions and respiratory spasms usually within 1 minute after the injection. If a larger dose, such as 0.5 cc , was injected rapidly into a rabbit of this size respiratory symptoms began in 20 seconds and death, accompanied by violent convulsions and spasms, followed within 10 seconds. For rabbits of 1,800 to 2,500 gm. the dose necessary to produce death was 0 4 cc. so that the reaction is in a measure quantitative

On examination of the rabbits immediately after cessation of the spasms, the heart was usually found beating rhythmically, although there were sometimes arrhythmias and fibrillation. All organs appeared normal, the lungs always being found collapsed

after opening the thorax   On opening the heart or vessels, larger ⁻
or smaller clots were always found and sometimes the whole blood
was found to be coagulated, especially if more than the minimum
fatal dose was given   In cases where the whole blood was not
clotted, the escaping fluid portion clotted more slowly than nor-‍-
mal, the length of time before the occurrence of spontaneous clot-
ting varying from a few minutes to a day.

These observations lead to the conclusion that in these cases
death was due in a large measure at least to intravascular clotting,
the symptoms probably resulting from the complete asphyxia of ⁻
the nervous system.   Although the death resembled that of
anaphylaxis, yet in contradistinction to the latter the'lungs were⁻
always found to collapse on opening the thorax, whereas in
deaths from anaphylaxis the lungs do not collapse.

I therefore undertook the study of the particular power of
lung tissue in causing thrombosis, as I was unable to find any
reference to the peculiar toxicity of lung extracts in this respect.
This study has resulted in showing the very remarkable throm-
boplastic power of the lungs, a power far surpassing that of any
other tissue of the body.

Intravascular coagulation from the intravenous injection of
tissue extracts has been known to be possible for many years.
Quoting from Carpenter (2),

"The contact of dead animal matter with the blood appears to promote
the coagulation of its fibrin in a very remarkable degree, occasioning
coagula to form, whilst it is yet actively moving in the vessels of the living
body   Thus M Dupuy found that the injection of cerebral substance into
the veins of an animal occasioned its death almost as instantaneously as
if prussic acid had been administered, the circulation being rapidly brought
to a stand, by the formation of voluminous clots in the heart and large
vessels.   These experiments were repeated and confirmed by M de Blain-
ville (Gazette Medicale, 1834, p 521) The same effect is produced
with still more potency, when the substance injected is rather undergoing
degradation, than actually dead, for it then seems to act somewhat after
the manner of a ferment, producing a marked diminution in the vitality
of the solids and fluids with which it may be brought in contact   Such
is pre-eminently the case with *pus*, as was long ago observed by Hunter
and as Mr H Lee has since determined more precisely   It was found by
the latter, that healthy blood received into a cup containing some offensive
pus coagulated in *two* minutes, whilst another sample of the same blood,
received into a clean vessel of similar size and shape, required *fifteen*
minutes for its complete coagulation "

Wooldridge (3), working from 1881 to 1889, was the first, however, to attempt to isolate the active thromboplastic material from the tissue extracts and to make a thorough study of the action of such extracts. He used extracts prepared from testes, thymus, and lymph glands, extracting the fresh tissues with water and precipitating the active material from the solution by making it strongly acid with acetic acid. The precipitate was then washed in water and dissolved in very dilute carbonate solution. Such solutions he found to produce thrombosis throughout the whole vascular system of rabbits after rapid intravenous injections, but in dogs, clots were usually found only in the portal system In any case, if the animal survived the thrombosis, a second injection within 24 hours was without effect, and blood drawn after such injection had a greatly diminished coagulability, spontaneous coagulation often being delayed as long as 24 hours The remarkable fact was discovered that the addition of more of the tissue extract to this blood outside the body produced coagulation in a few minutes. He observed that the degree of non-coagulability of the blood was in a measure proportional to the extent of the thrombosis

In studying the extracts to determine the active substance, he decided that a protein-phospholipin compound was responsible for the results. This compound could be extracted from the acetic acid precipitate with dilute alkali He states that the solution was not a true solution since the dissolved substance would not pass through a clay cell. If he extracted this precipitate with alcohol and ether the activity of the undissolved residue was lost, so that the phospholipin must be a necessary part of the compound. Also on digestion of the solution with pepsin and hydrochloric acid, the phospholipin, with a small amount of the protein, was precipitated, and the remaining solution had lost its activity. The precipitate was active. Examination of the phospholipin convinced him it was a lecithin-like substance, although purified lecithin from egg yolk was not active. Lecithin prepared from other tissues gave him the same results as the tissue extracts Wooldridge was not familiar with cephalin at that time, so that he did not identify the active phospholipins as cephalin which was present as an impurity in his tissue lecithin, although he did show that not all lecithin prepa-

ratrons were active.   More recent work in Howell's laboratory
(4) indicated that the phospholipin inducing coagulation dis-
covered by Wooldridge is cephalin, but the strange fact appeared
from my experiments that although the nervous tissue is the
tissue believed to be the richest in cephalin, yet extracts of the
brain made in the same way as these lung extracts did not cause
intravascular coagulation and death in rabbits in doses up to 3
cc for an 1,800 gm. rabbit   It was therefore deemed advisable
to compare as quantitatively as possible the thromboplastic
activity of lung tissue extracts with that of extracts of other
tissues of the body

Fresh tissues of dogs killed in ether anesthesia and rabbits
were used in making extracts similar to the lung extract
described above, that is, for every gm. of the fresh tissue, after
grinding thoroughly with sand, 10 cc of 0 9 per cent NaCl
solution were added, mixed well, and centrifuged to remove all
solid particles   Extracts thus made were found to undergo a
gradual loss of their power to induce coagulation when injected
into the circulation or of hastening coagulation when added to
blood outside the body   When standing at 5°C very little change
occurred in the first few days, but activity had almost disap-
peared at the end of 3 weeks   This loss of activity was not due
to a settling out of the active matter formerly in suspension,
since on shaking and injecting, or adding precipitate and solution
mixed, the diminution of activity persisted.   It might be, how-
ever, that a gradual agglomeration of particles had occurred
leading to a smaller number of larger aggregates with a resulting
diminution of surface and hence of activity.   This rather than
a chemical change might explain the progressive loss of power.
This possibility will be further investigated.

The activity of the tissue extracts prepared as described were
tested in two ways, (1) by injections into the blood stream of
dogs and rabbits, and (2) by testing their power of hastening
coagulation when added to peptone and oxalate plasma outside
the body.   In the latter case the test-tube method for determin-
ing the coagulation time was used, coagulation being considered
complete when the tube could be inverted without spilling the
contents   If care was taken in shaking the tubes and in keeping
all other factors constant, this method was considered sufficiently
accurate for this work.

Peptone plasma and oxalated plasma of dog's blood were used in most of the tests, although two sets of rabbit tissue extracts were tested on rabbit blood rendered partially non-coagulable by a process which will be described in another paper.

In order to compare the activity of the extracts of the different tissues, one method was to find the amount of the various extracts necessary to induce coagulation of 1 cc of peptone plasma in the same time as a definite amount of lung extract. Another method used was to add the same amount of the different extracts to oxalate plasma and compare the amount of acceleration of coagulation of the plasma by serum. All tests were carried out in a water bath at 38–40°C

Tables I and II indicate the results of the tests carried out with the extracts of the tissues of two different dogs.

The pancreas, skeletal muscle, thyroid, and omentum contained so little of the thromboplastic material that they would not accelerate the coagulation to 30 seconds in any amount, so the figures for them are approximations derived from the degree of lessening of coagulation time by increasing amounts of the extracts.

TABLE I

Tissue extract used (dog)	Time of coagulation of 1 cc of oxalate dog plasma, by 3 drops of extract and 6 drops of serum *	
	Tissues of Dog I	Tissues of Dog II.
	min	*min*
Lung	$\frac{1}{2}$	$\frac{1}{2}$
Kidney	4	4
Testes	7	6
Brain	$8\frac{1}{2}$	6
Heart	8	7
Spleen	9	4
Bone marrow	7	7
Adrenal	8	$8\frac{1}{2}$
Liver	11	8
Omentum	9	16
Skeletal muscle	14	12
Pancreas	14	14
Thyroid	14	15

* 1 cc of oxalate plasma and 6 drops of serum (with no extract) showed no coagulation in $1\frac{1}{2}$ hours, but were coagulated in 24 hours

TABLE II

Tissue extract used (dog)	Amount of extract necessary to coagulate 1 cc of peptone dog plasma in 30 seconds	
	Tissues of Dog I	Tissues of Dog II.
	gtt	*gtt*
Lung	2	2
Kidney	5	5
Testes	8	15
Brain	7	6
Heart	20	12
Spleen	4	4
Bone marrow	20	20
Adrenal	6	20
Liver	25	20
Omentum	30 +	30 +
Skeletal muscle	30 +	30 +
Pancreas	40 +	30 +
Thyroid	30 +	20 +

The activity of extracts of the tissues of two rabbits on partially non-coagulable rabbit blood is shown in Table III.

TABLE III

Tissue extract used (rabbit)	Amount of extract necessary to clot 1 cc of rabbit blood	
	90 seconds Tissues of Rabbit I	70 seconds Tissues of Rabbit II.
	gtt	*gtt*
Lung	1	1
Kidney	5	5
Heart	5	5
Thymus		3
Spleen	8	4
Brain	8	13
Skin		10
Testes		20 +
Ovary	22	
Uterus	20	
Liver	30 +	30
Bone marrow		20 +
Pancreas		20 +
Adrenal	26 +	20 +
Omentum	30	
Skeletal muscle	30 +	30 +

Here again some of the tissues were so poor in the active substance that they would not clot the blood in the specified time in any amount, so that the figures given are only approximations.

In every case studied the lung extract was found to be much stronger than that from any other tissue, being from two to thirty times as strong as the other tissues in accelerating coagulation. Kidney, heart, brain, spleen, thymus, and skin come next in activity, somewhat in the order named. Then, certain other tissues, pancreas, skeletal muscle, liver, bone marrow, omentum, and adrenal, showed only slight thromboplastic activity as compared to the lung tissue.

Having thus established the predominant thromboplastic power of the lung extracts I next attempted to discover whether the toxicity from intravenous injections of these extracts paralleled their thromboplastic activity. To this end injections were made into rats and rabbits In using rats the injections were made directly into the heart, but with rabbits injections were into an ear vein. Adult white rats, weighing about 350 gm., were used. They were found to be much less affected by partial coagulation of the blood in the vessels than rabbits, quick death being produced only by almost solid intravascular coagulation following injections of relatively large doses of the extracts Most of the extracts of rabbits and rat tissues would not produce coagulation in rats in doses up to 3 cc. when made up in the usual way, so double strengths of these extracts were used, that is, only half the usual amount of saline solution was used for each gm of fresh tissue. These extracts were labeled (×2). Table IV indicates the dosages of the various extracts necessary to produce rather extensive coagulation in the vessels of the rats.

In intravenous injections into rabbits only two of the tissue extracts proved fatal, death occurring in rabbits after only slight thrombosis. Lung extract was fatal in doses of 0 3 to 0.4 cc and kidney extract (×2) was fatal in a dose of 2 0 cc. producing only a slight amount of thrombosis, and with death occurring after a delay of 2½ minutes. None of the other tissue extracts was fatal in doses up to 3.0 cc. Wooldridge reports solid thrombosis of the whole system in rabbits with injections of thymus, testes, and lymph gland extracts, but he used many times the amount of material for injections that was used in these cases.

Thus he used $\frac{1}{2}$ to 1 gm of the washed acetic acid precipitate from the extracts to obtain such solid intravascular coagulations.

From these intravenous injections into rats and rabbits as described above, it is evident that the extracts are toxic in much the same order as they exhibit thromboplastic activity in extravascular coagulation, lung extract being much the most active of all tissue extracts in each case

Most hemostatic preparations on the market today are prepared from brain material, but from the results of the above experiments it would seem that lung tissue would afford a much stronger hemostatic   Battelli (5) describes a method for preparing such material from fresh horse or sheep lungs.

TABLE IV

Extract used	Coagulative dosage for rats
	cc
Rat lung extract	0 5
" kidney "	1 0
" " " (×2)	0 9
" brain " (×2)	1 1
Rabbit lung extract	0 4
" kidney " (×2)	0 9
" brain "	3 0
" " " (×2)	1 0
" heart " (×2)	1 5
" liver " (×2)	2 0

The significance of such a strong thromboplastic activity in lung tissue, and not so very much less in kidney tissue, is an interesting question   Is it a protective mechanism against possible hemorrhages in these organs in which the rich capillary network is covered only by a one celled layer of epithelium? This high activity of the lung tissue may perhaps have an especial function in certain diseases, such as pneumonia, where there occurs such an extensive destruction of lung tissue. With the extremely rich blood supply in all parts of the lungs one would expect severe hemorrhages in these cases, but the hemorrhages are, in fact, not one of the main characteristics of this disease. May it not be the liberation of this active material by the tissue destruction which protects against extensive hemorrhages? The

one extract of the skin tested was also found to possess considerable thromboplastic activity, again denoting a possible protective mechanism

The investigation of the nature of the active principle in lung extract is being carried further and will form the subject of another paper.

SUMMARY.

1. Sudden death from the intravenous injection of lung tissue extract is apparently due to intravascular coagulation, although there is a possibility of anaphylactic effect on the lungs, not sufficient, however, to keep them from collapsing on opening the thorax.

2. Tissue extracts were tested as to their thromboplastic activity on the blood both intra- and extravascularly. Lung extracts were found far more active than the extracts of any other tissue, kidney coming second, and then heart, brain, spleen, thymus, testes, skin, somewhat in the order named. The remaining tissues were weakly active as compared to lung, some of them showing very slight thromboplastic action.

3. Lung tissue offers a possible source for the preparation of a strong hemostatic.

4. The strong coagulative activity of lung and kidney tissues, and to a lesser degree of skin, is suggestive of a possible protective mechanism against hemorrhage.

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THE HYDROLYSIS OF STIZOLOBIN, THE GLOBULIN OF THE CHINESE VELVET BEAN, STIZOLOBIUM NIVEUM.

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The preparation of the globulin used for this hydrolysis is described in a previous publication from this laboratory (1)

The hydrolysis and the determination of the resulting amino-acids were carried out, for the greater part, in the usual manner with a few exceptions which will be noted later

A slight modification of the customary method of drying the ether solutions of the esters with anhydrous sodium sulfate was made. It seems very probable that during the extraction of the esters with ether, after their liberation with sodium ethylate, or with sodium hydroxide, as is sometimes necessary after the second esterification, some of the alkali passes into the ether, and later decomposes to some extent the easily hydrolyzed esters. Accordingly, finely powdered ammonium chloride was mixed with the sodium sulfate used for drying the ether solution of the esters. Any caustic alkali present would thus be removed by the action of ammonium chloride, with the formation of sodium chloride and ammonia, and in this way the hydrolysis of the esters reduced to a minimum

The method of Levene and Van Slyke (2) for the separation of alanine from valine by means of phosphotungstic acid was used with good results.

The percentages of basic amino-acids in stizolobin, as determined by the Van Slyke method, have already been published (1).

The percentages of the various amino-acids obtained by the hydrolysis of stizolobin are summarized in Table I For the sake of comparison, the percentages of amino-acids found in phaseolin,

the globulin of the white navy bean, *Phaseolus vulgaris*, are also given.

As is seen, the summation of the hydrolysis of stizolobin, although comparatively high, falls far short of accounting for the theoretical amount of hydrolysis products which should result. Some time ago we became convinced that with the methods now in use one cannot hope to account quantitatively for nearly all

TABLE I

Percentage of Amino-Acids in Stizolobin and Phaseolin

	Stizolobin	Phaseolin *
	per cent	*per cent*
Glycine .	1 66	0 6
Alanine .	2 41	1 8
Valine	2 88	1 0
Leucine . .	9 02	9 7
Proline .	4 00	2 8
Phenylalanine	3 10	3 3
Aspartic acid	9 23	5 3
Glutaminic acid	14 59	14 6
Hydroxyglutaminic acid	2 81	
Serine . .	0 67	0.4
Tyrosine .	6 24†	2 2
Cystine	1 13	
Arginine..	7 14	4 9
Histidine. .	2 27	2 0
Lysine . .	8 51	4 0
Tryptophane .	Present	
Ammonia	1 55	2 1
Total .	77 21	54 7

* Osborne, T. B , and Clapp, S H , *Am J. Physiol* , 1907, xviii, 295.
† By the colorimetric method of Folin and Denis

the products which proteins yield on hydrolysis, but that methods must be developed whereby the products of hydrolysis can be separated more directly than by esterifying the amino-acids and distilling the esters as originally worked out by Fischer. As is well known, it is rarely if ever a 100 per cent esterification of a pure organic acid can be accomplished, and when one considers the complex mixture which results from the hydrolysis of a protein, consisting of eighteen or more amino-acids, humin, and other

secondary decomposition products, a high percentage esterification can hardly be expected. The hope of attaining anywhere near a theoretically complete summation is therefore practically excluded from the beginning. We have been working for some time on the direct determination of the amino-acids in another protein by methods which do not involve the process of esterification and distillation of esters. This work is still in progress.

A method for the determination of proline without involving the esterification of the amino-acids is described. The products of hydrolysis of the protein, after having first removed the bases and mineral acids, are obtained in a dry powdered form, from which the proline is extracted with boiling absolute alcohol. The non-amino nitrogen in the solution is determined and the per cent of proline calculated. Several determinations made in this way gave results closely agreeing with each other, and also with the percentage obtained from the esters.

Since the work on this hydrolysis was completed an article published by Dakin (3) appeared in which he describes a method for the partial separation of the amino-acids in a hydrolysis mixture by extracting the aqueous solution of amino-acids with butyl alcohol, and in which is also described for the first time the isolation and properties of the new amino-acid, hydroxyglutaminic acid. By means of this method, Dakin states that the products of hydrolysis of a protein may be readily separated almost completely into the following five groups.

(1) Monoamino-acids, both aliphatic and aromatic, insoluble in alcohol but extracted by butyl alcohol.

(2) Proline, soluble in alcohol and extracted by butyl alcohol.

(3) Peptide anhydrides (diketopiperazines), extracted by butyl alcohol but separated from (2) by sparing solubility in alcohol or water.

(4) Dicarboxylic acids, not extracted by butyl alcohol.

(5) Diamino-acids, not extracted by butyl alcohol but separable from (4) by phosphotungstic acid and other means.

In order to check up by this method the results we had already obtained for aspartic and glutaminic acids in stizolobin by the ester method, as well as to look for hydroxyglutaminic acid, some of the residues of amino-acids remaining from the direct determination of proline were examined by this method for

aspartic, glutaminic, and hydroxyglutaminic acids. 2 81 per cent of hydroxyglutaminic acid was obtained in the form of the difficultly soluble silver salt.

One of the most striking results of this examination is the extraordinarily high percentage of aspartic acid obtained; namely, 9.23 per cent. As the amount obtained from the esters was only 5.70 per cent, it is highly indicative that the percentages of aspartic acid obtained in previous hydrolyses of proteins do not nearly represent the actual amount present.

The amount of glutaminic acid obtained by this method was somewhat lower than that obtained from the large hydrolysis, due to the fact that some of this amino-acid had been previously removed in the form of pyrrolidonecarboxylic acid, while making the proline determination.

The method outlined by Dakin will doubtless prove of great value in the further development of methods for the direct separation and determination of the products of hydrolysis of proteins.

<center>EXPERIMENTAL.</center>

A quantity of stizolobin, equivalent to 405 gm of the ash and moisture-free protein, was hydrolyzed in two separate portions of 200 and 240 gm each, with 1,300 cc. of hydrochloric acid (specific gravity 1.1), by first heating on a steam bath until nearly all the protein had dissolved. The hydrolysis was then continued by boiling the solutions in an oil bath for 30 hours. The solutions were then concentrated to about one-half their original volumes, and after removal of most of the color by treatment with norite, were saturated, cold, with dry hydrochloric acid gas. After standing for about a week at nearly 0°, the glutaminic acid hydrochloride was removed by filtration. There were finally obtained, after removal of ammonia with barium hydroxide in the usual way, 27 23 and 24 83 gm , respectively, of glutaminic acid hydrochloride, equivalent to a total of 41 72 gm. of the free acid. This amount, together with the 17.28 gm subsequently isolated from the esters, is equivalent to 14.59 per cent of the stizolobin.

The glutaminic acid hydrochloride decomposed with effervescence at 197° The free acid. obtained by decomposing the

hydrochloride with an equivalent amount of normal potassium hydroxide, decomposed at 202° and was analyzed with the following results:

0 1491 gm of substance gave 0 2249 gm of carbon dioxide and 0.0872 gm of water

	Calculated for $C_5H_9O_4N$.	Found
C	40 80	41 14
H	6 12	6 54

The filtrates and washings from the above direct determination of glutaminic acid hydrochloride were united and concentrated to a heavy syrup. This residue was freed from water by repeated evaporations with alcohol under reduced pressure and esterified in the usual way, with the exception that no zinc chloride was used. After liberating the esters with sodium ethylate the sodium chloride was removed by centrifugation and the alcohol distilled from the esters under reduced pressure The alcohol was reserved for further examination. On stirring the syrupy residue of esters with ether nearly all dissolved, but on dilution with more ether a flocculent precipitate separated which soon settled and formed a sticky, viscous layer. The addition of ether was continued until only a slight turbidity was produced. The ether extracts were filtered and dried in the usual way over anhydrous sodium sulfate

The sodium chloride obtained from the alcoholic solution of the esters was dissolved in water and the solution saturated with hydrochloric acid gas. The salt which separated was removed by filtration. To the filtrate was added the above mentioned sticky residue which did not dissolve in the ether when extracting the esters. The solution was then subjected to a second esterification. The residue of ester hydrochlorides left after removing the alcohol by distillation was very viscous. It was therefore dissolved in enough absolute alcohol to form a thin syrup. After cooling to 0° the esters were liberated by the cautious addition of an aqueous 50 per cent solution of sodium hydroxide. The esters were extracted with ether and dried over anhydrous sodium sulfate to which was added a little powdered ammonium chloride.

The ether was removed from the esters by distillation at atmospheric pressure 332 gm. of esters were obtained, 128 gm of which were obtained from the second esterification.

Alcohol Distilled from the Esters —The alcohol which had been distilled from the esters was acidified with hydrochloric acid and the solution evaporated to dryness under reduced pressure. The residue of ester hydrochlorides was hydrolyzed by boiling with dilute hydrochloric acid, and the solution evaporated to dryness. After expelling ammonia by boiling with barium hydroxide, and subsequent removal of barium and hydrochloric acid, there were obtained 8 48 gm. of amino-acids. The amino-acids insoluble in absolute alcohol weighed 6 66 gm Both the proline and the other amino-acids were added to the corresponding portions of Fraction I of the distilled esters

Ether Distilled from the Esters —The ether distilled from the esters was strongly acidified with a dry alcoholic solution of hydrochloric acid, and allowed to stand at nearly 0° for several weeks The white crystalline product which separated was boiled with barium hydroxide to remove ammonia. The barium and hydrochloric acid were then quantitatively removed, and after concentrating the solution to a small volume 3.81 gm of glycine separated, which, for identification, was converted into its ethyl ester hydrochloride The latter crystallized from alcohol in the characteristic long prisms or needles which melted sharply to a clear oil at 144°.

0 1987 gm of substance required 14 15 cc of 0 1 N sulfuric acid

		Calculated for $C_4H_{10}O_2NCl$	Found
N	.	10 04	10 03

The glycine thus obtained, together with that subsequently isolated from the distilled esters, amounted to 6 72 gm or 1 66 per cent of the protein

The amino-acids present in the form of their esters in the original ether filtrate from the glycine ester hydrochloride were regenerated and weighed 9 24 gm , of which 6 88 gm were insoluble in absolute alcohol The latter and the alcoholic extract of proline were added to similar portions of Fraction I of the distilled esters.

The esters obtained after distilling off the ether were separated into the following fractions in the usual way by distillation under reduced pressure

Fraction	Temperature of the bath	Temperature of the vapors	Pressure	Weight
	°C	°C	mm	gm
I	100	50	35	21
II	113	85	3	85
III	135	107	3	61
Distillation residue				131
Contents of the liquid air tube				10

The contents of the liquid air tube were made strongly acid with hydrochloric acid and hydrolyzed by boiling for several hours. After expelling the ammonia with barium hydroxide and subsequent removal of barium and hydrochloric acid there were obtained 2 87 gm of amino-acids, of which 2.04 gm. were insoluble in absolute alcohol. The latter were added to Fraction I.

Fraction I.—The esters of this fraction, which also contained some alcohol, were hydrolyzed in the usual way by boiling with water. There were obtained 4 64 gm of amino-acids, 3.73 gm. of which were insoluble in absolute alcohol To the latter were added the alcohol-insoluble amino-acids obtained from the following sources: (1) The alcohol distilled from the esters, 6.66 gm , (2) the ether distilled from the esters, 6.88 gm , (3) the contents of the liquid air tube, 2.04 gm , (4) the precipitate which separated on standing from the united alcoholic extracts of proline, 2.30 gm From this mixture were isolated by means of the lead salts, 5 62 gm. of leucine and 2 64 gm. of valine. The leucine had the following composition

0 1825 gm of substance gave 0 3678 gm of carbon dioxide and 0 1671 gm of water

			Calculated for $C_6H_{13}O_2N$	Found
C			54 96	54 96
H			9 99	10 24

Analysis of the valine gave the following results·

0 1495 gm of substance gave 0 2828 gm of carbon dioxide and 0 1319 gm of water

			Calculated for $C_5H_{11}O_2N$	Found
C			51 28	51 59
H			9 47	9 80

The filtrates from the above leucine and valine were united, the amino-acids esterified and the resulting product was examined for glycine.   There were obtained 5.0 gm. of glycine ester hydrochloride, equivalent to 2.69 gm. of glycine   The glycine ester hydrochloride crystallized from alcohol in the characteristic needles which melted sharply at 144°C.

The filtrate from the glycine ester hydrochloride, after having quantitatively removed the hydrochloric acid, yielded 7.37 gm. of alanine which crystallized in needles and prisms.

0 2028 gm of substance gave 0 3004 gm of carbon dioxide and 0.1377 gm of water.

						Calculated for $C_3H_7O_2N$	Found
C	.	.	.	. .	.	40 41	40 40
H	.			.	. . .	7 92	7 54

A small amount of glycine (0 22 gm.) was further separated (4) from the filtrate from the alanine, in the form of its picrate, which decomposed with effervescence at 195°C.

Fraction II.—This fraction yielded 52 gm. of amino-acids of which 42 6 gm   were insoluble in absolute alcohol.   By direct fractional crystallization 16.50 gm. of leucine and 3 29 gm. of valine were obtained   Analysis of the leucine showed it to have the following composition:

0 1459 gm of substance gave 0 2937 gm. of carbon dioxide and 0 1313 gm of water

			Calculated for $C_6H_{13}O_2N$	Found
C			54 96	54 90
H	.	.	9 99	9 99

The valine gave the following results on analysis:

0 1666 gm of substance gave 0 3151 gm of carbon dioxide and 0 1426 gm of water

			Calculated for $C_5H_{11}O_2N$	Found
C	.		51 28	51 58
H		. . .	9 47	9 51

A mixture of leucine and valine, after subjection to Levene and Van Slyke's lead salt method of separation, yielded 14 44 gm. of leucine and 2 43 gm of valine.

A fraction was further obtained consisting of a mixture of alanine and valine. The amino-acids of this mixture were separated by means of phosphotungstic acid according to Levene and Van Slyke's method (2)   3 32 gm of valine and 2 38 gm of alanine were thus obtained.

All the alcoholic extracts of proline were united and allowed to stand for several days   The precipitate which had separated, after having been filtered off and washed with absolute alcohol, weighed 2 30 gm., and was added to the amino-acids of Fraction I. The clear solution was then evaporated to dryness under reduced pressure   The residue was completely soluble in cold absolute alcohol. The solution was again evaporated to dryness and the residue dissolved in water and made up to a volume of 500 cc   The total nitrogen in the solution was 2 6023 gm , and the amino nitrogen 0.7399 gm   The difference, or non-amino nitrogen, 1.8623 gm. corresponds to 15.30 gm of proline, equivalent to 3 77 per cent of the proline   Special experiments for the direct determination of proline are described

Fraction III.—Phenylalanine ester was separated from this fraction in the usual way by extraction with ether   After hydrolyzing the ester with hydrochloric acid, and decomposing the hydrochloride with ammonia, 4 16 gm of phenylalanine were obtained.

0 1531 gm of substance gave 0 3660 gm of carbon dioxide and 0 0948 gm of water

	Calculated for $C_9H_{11}O_2N$	Found
C	65 45	65 20
H	6 66	6 98

The esters remaining after the removal of the phenylalanine were hydrolyzed with barium hydroxide, and yielded 2.92 gm of aspartic acid as the barium salt, and 15 80 gm in the form of the copper salt. The free aspartic acid gave the following results on analysis

0 1664 gm of substance gave 0 2200 gm of carbon dioxide and 0 0825 gm of water

	Calculated for $C_4H_7O_4N$	Found
C	36 09	36 06
H	5 26	5 51

The copper aspartate was analyzed with the following results.

0 2503 gm. of substance (air-dried) gave 0 0717 gm of copper oxide

	Calculated for $C_4H_5O_4NCu\ 4\frac{1}{2}H_2O$	Found
Cu	23 07	22 90

There were also obtained 2.73 gm of a substance which after several recrystallizations from water separated in the form of rather indefinite, microscopic, octagonal plates Analysis showed these crystals to contain 35 20 per cent carbon, 7.00 per cent hydrogen, and 12 47 per cent nitrogen. The small amount of the substance in hand, and its high degree of solubility, prevented further examination of this material Its solubility, crystalline form, sweetish taste, and composition, as above noted, leave little or no doubt that this substance was chiefly serine. Especially significant in supporting this conclusion is the high percentage of nitrogen found, as serine contains nearly 3 per cent more nitrogen than any of the other amino-acids usually found as products of protein hydrolysis, except proline Serine theoretically contains 34 29 per cent carbon, 6 67 per cent hydrogen, and 13 33 per cent nitrogen.

Distillation Residue.—The residue remaining after the distillation of the esters was dissolved in water and shaken with ether to separate the phenylalanine, of which 8.41 gm were obtained.

The aqueous layer was concentrated somewhat and sufficient concentrated hydrochloric acid added to make the solution about 25 per cent. This solution was boiled for 12 hours in order to hydrolyze any pyrrolidonecarboxylic acid that might be present. It was then concentrated to a small volume, and saturated cold with hydrochloric acid gas After standing for a long time at 0°, 17.52 gm. of glutaminic acid hydrochloride were isolated It decomposed with effervescence at 197°. The filtrates from the glutaminic acid hydrochloride were united and the bases removed with phosphotungstic acid. From the resulting solution, after removing the hydrochloric and phosphotungstic acids, there were further obtained 4.4 gm of aspartic acid and 3 24 gm of glutaminic acid

Tyrosine—Tyrosine was determined by the colorimetric method of Folin and Denis (5, 6). 1 gm. of stizolobin was boiled

for 12 hours with 20 per cent hydrochloric acid.   The tyrosine was found to be equivalent to 6.24 per cent of the protein

As the percentage of tyrosine obtained by this method is usually 2 or 3 per cent higher than that obtained by direct isolation of the amino-acid, due to the extreme difficulty of separating all the tyrosine from the other products of hydrolysis, a direct determination of tyrosine was made for comparison   50 gm. of the protein were accordingly hydrolyzed with hydrochloric acid, and as much as possible of the latter was removed by distillation under reduced pressure   The calculated amount of normal potassium hydroxide to react with the chlorine remaining in the residue was then added.   There were obtained from the resulting solution by careful fractional crystallization, 2.42 gm of tyrosine, equivalent to 5.25 per cent of the protein.   That not all the tyrosine was removed even then was indicated by the fact that the mother liquors still gave a strong positive test with Millon's reagent.

The tyrosine which was isolated gave the following results on analysis·

0 2036 gm of substance required 11 2 cc of 0 1 N HCl ´

	Calculated for $C_9H_{11}O_3N$	Found
N	7 73	7 72

Proline.—Several determinations of proline were made directly without involving the ester method of Fischer.   The following procedure gave consistent results, which closely agreed with the percentage obtained by the ester method 10 gm. of the protein are hydrolyzed by boiling with 20 per cent hydrochloric acid for about 30 hours.   The solution is then concentrated under reduced pressure to a thick syrup, and the residue dissolved in water.   After filtering off the suspended humin, the solution is made up to 200 cc containing 3 5 per cent hydrochloric acid, and phosphotungstic acid added until all precipitation has ceased. After standing for 48 hours the precipitate is filtered off and washed in the usual way.   Phosphotungstic acid is then removed from the filtrate by means of ether and amyl alcohol, and the solution of amino-acids boiled with a slight excess of barium hydroxide in order to remove any ammonia or phosphotungstic

acid that might be present.   After filtering, the excess of barium
is removed quantitatively with sulfuric acid and the solution
made slightly alkaline with sodium hydroxide, in order to neu-
tralize the hydrochloric acid present   The solution is then re-
acidified with acetic acid and evaporated to dryness, and the dry,
finely powdered residue of amino-acids extracted by boiling with
absolute alcohol for about $1\frac{1}{2}$ hours.   The alcoholic extract is
acidified with hydrochloric acid and the alcohol removed by
distillation under reduced pressure.   The residue is then boiled
for 10 to 15 hours with 25 per cent hydrochloric acid to regenerate
any glutaminic acid from pyrrolidonecarboxylic acid which may
have been formed during the preceding operations   The solution
is then freed from hydrochloric acid as completely as possible by
distillation under reduced pressure, the residue dissolved in water
and made slightly alkaline with sodium hydroxide, and then
reacidified with acetic acid   The resulting solution is made up to
100 cc and amino nitrogen and total nitrogen are determined in
10 cc aliquot portions   From these data the amount of proline
is calculated   Eight determinations of proline made in this way
gave closely agreeing results the average of which was 4 per cent.

   The Di-Basic Amino-Acids —As the residues of the amino-
acids remaining from some of the direct proline determinations
were on hand they were examined for the di-basic amino-acids
according to the method of Dakin (3)   Four of these residues,
which represented 40 gm. of stizolobin, were united and used
for the analysis   The proline, ammonia, bases, and most of the
tyrosine had been previously removed.   The amino-acids were
dissolved in about 600 cc of water and extracted with butyl
alcohol, according to Dakin's directions   The extraction was
continued for about 38 hours, at the end of which time there
were practically no more acids being carried over by the butyl
alcohol.   As the amount of material available for this extraction
was too small for a complete analysis, the monoamino-acids
which had been carried over by the butyl alcohol were not further
examined   The aqueous solution which had been extracted was
examined for glutaminic acid in the usual way   4.41 gm of
glutaminic acid, isolated as the hydrochloride, were obtained.
This corresponds to 11 02 per cent of the protein.   This per-
centage is somewhat lower than that which we obtained from

the main hydrolysis. This was to be expected, since some of the glutaminic acid was lost during the extractions for proline by being converted into pyrrolidonecarboxylic acid   The proline extract was always boiled with 25 per cent hydrochloric acid before determining amino nitrogen, and it was always found that some of the glutaminic acid had been converted into pyrrolidone-carboxylic acid.

The amino-acids in the filtrate from the glutaminic acid hydrochloride were converted into their calcium salts, according to Foreman's method (7), and the aspartic acid was isolated as the copper salt   An unusually large amount of the copper salt separated in the characteristic crystalline form   The air-dried salt weighed 7 69 gm , and without recrystallizing gave the following results on analysis.

0 2082 gm of substance gave 0 0602 gm of copper oxide

		Calculated for $C_4H_5O_4NCu\ 4\frac{1}{2}H_2O$	Found
Cu	..	23 07	23 10

The above amount of copper aspartate is equivalent to 3.69 gm. of the free acid which is 9 23 per cent of the protein.   This is 3 53 per cent more than was obtained from the esters.

Copper was removed from the filtrate from the copper aspartate, and the solution examined for hydroxyglutaminic acid by precipitating the acid in the form of its silver salt, according to Dakin's method   There were obtained 2 63 gm. of the difficultly soluble silver salt, which is equivalent to 1.12 gm of hydroxyglutaminic acid, or 2.81 per cent of the protein

0 2378 gm of substance gave 0 1803 gm of silver chloride

	Calculated for $C_5H_7O_5NAg_2$	Found
Ag	57.26	57 07

SUMMARY.

The globulin of the Chinese velvet bean has been hydrolyzed and the percentages of the resulting amino-acids determined (see Table I)

A modification of the usual method of determining proline is described

Aspartic acid was determined both by the usual ester method. and by the method, recently published by Dakin, of extraction of the solution of amino-acids with butyl alcohol. The yield obtained by the former method was 5 70 per cent, while by the latter method the unusually high percentage of 9 23 per cent was obtained. By means of the extraction method 2 81 per cent of hydroxyglutaminic acid was also obtained.

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2 Levene, P A , and Van Slyke, D D , *J Biol Chem* , 1913–14, xvi, 103.
3 Dakin, H D , *Biochem J* , 1918, xii, 290
4 Levene, P A , and Van Slyke, D D , *J Biol Chem* , 1912, xii, 285
5. Folin, O , and Denis, W , *J Biol Chem.*, 1912, xii, 245
6 Johns, C. O , and Jones, D B , *J. Biol Chem* . 1918, xxxvi, 319
7 Foreman, F W., *Biochem J* , 1914–15, viii, 463

THE NUTRITIVE VALUE OF THE BANANA. II.

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Introduction and Object of the Investigation

It has been shown in a preceding article[1] that bananas alone as a food do not produce growth of young albino rats   The addition of 16 per cent of purified casein, which supplied the protein deficiency of the food, and 0 5 per cent of yeast preparation, or of aqueous or alcoholic extract of fresh carrots, which supplemented the shortage of water-soluble accessory substance, constituted a complete diet for the growth, maintenance, and reproduction of albino rats.   Such a diet was not adequate, however, for the production of proper milk by the mother.   We stated that the addition of a small amount (10 cc) of cow's milk to the mother's diet from the time of birth of the young until they are weaned is absolutely imperative, if the young are to survive and to maintain normal growth.

Further experiments on this point are shown in Tables I, II, and III, from which it is clearly seen that the new-born whose mothers were fed with a ration made up of bananas, casein, and yeast failed to grow and died from starvation, or were killed by the mother shortly after they were born, except in one instance where the young were reared but their body weights were very much below normal   The mother of these lost 10 gm of her body weight during 1 month of lactation.   On the other hand, the addition of cow's milk to the same diet made it a proper diet for the production of suitable milk during the period of lactation   The mothers maintained their body weights on this diet, and Rats 49 and 219 gained in body weight   The health and condition of these animals appeared normal in all respects

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[1] Sugiura, K , and Benedict, S R , *J. Biol. Chem* , 1918, xxxvi, 171.

The aim of the present investigation has been to study the unknown chemical substance or substances present in milk, which are indispensable for the growth of young animals during the period of lactation.

Review of Previous Investigations.

Of all foods, milk is the most important   It contains all the nutrients essential for growth   The important constituents of milk are water, fats, casein,[2] lactalbumin,[3,4] lactoglobulin,[4,5] lactose, lecithin,[6] cholesterol,[7] urea,[8] ammonia,[9] the purine bases, alcohol-soluble protein,[10] inorganic salts, enzymes, and unidentified accessory factors [11,12]   Many foreign substances, such as flavors, condiments, and stimulants, introduced with the food, are secreted in the milk

A brief review of the more characteristic investigations relating to milk production of adult animals and to the subsequent growth of young may throw light on the nature of the catalytic substances present in milk

Decaisne,[13] during the siege of Paris, made an observation upon the milk production of forty-three women and the behavior of their infants   Twelve strong, healthy women had plenty of milk of good quality and their children obtained enough milk at the expense of catabolized tissue of the mothers   Fifteen women had little milk of poor quality   Their children became weak and had enteritis   Sixteen women had very little milk and more than three-fourths of the children died from starvation   Most of the forty-three women appeared to be suffering from malnutrition   Milk analyses revealed that the amount of fats, casein, milk-sugar, and salts was diminished while albumin increased as a result of insufficient nutrition

McCollum and Davis[14] have shown that young rats maintained normal growth for periods of 70 to 120 days on a ration of purified food substances

[2] Vernois, A.-G.-M., and Becquerel, A., *Ann Hyg*, 1853, xlix, 257, 1, 43

[3] Sebelien, J, *Z physiol Chem*, 1885, ix, 445

[4] Halliburton, W D, *J Physiol*, 1890, xi. 448

[5] Sebelien, J, *J Physiol*, 1891, xii, 95

[6] Stoklasa, J, *Z physiol. Chem*, 1897, xxiii, 343.

[7] Filia, A, *Riv clin pediat*, 1914, xii, 339

[8] Schondorff, B, *Arch. ges Physiol*, 1900, lxxxi, 42

[9] Sherman, H C, Berg, W N, Cohen, L J·, and Whitman, W G, *J Biol Chem*, 1907, iii, 171

[10] Osborne, T B, and Wakeman, A J, *J Biol Chem*, 1918, xxxiii, 7

[11] Osborne, T B, and Mendel, L B, *Carnegie Institution of Washington, Publication No 156*, 1911, pts. 1 and 2

[12] Hopkins, F G, *J Physiol*, 1912, xlv, 425

[13] Decaisne, E, *Gaz méd*, 1871, xxvi, 317

[14] McCollum, E. V, and Davis, M, *J Biol Chem*, 1913, xv, 167.

consisting of casein, carbohydrate, and salt mixtures or the same ration in which a part of the carbohydrates was replaced by lard   Upon these rations females did not become pregnant, however, upon the addition of 1 gm of ether extract of egg or butter, not only was growth resumed but they also became pregnant and gave birth to young   Some of the young animals were eaten by their mothers, others were reared but were very much undersized   The cause of the subnormal growth of young was due to the insufficient milk production of the mothers

Osborne and Mendel,[15] working with young white rats, found that the milk food, which consisted of milk powder, 60 per cent, starch, 12 per cent, and lard, 28 per cent, was an adequate diet and animals not only had grown from infancy to full maturity, but also gave birth to young which in turn thrived upon the same diet

Hart and Humphrey,[16] using two grades of Holsteins, showed that the nitrogen of alfalfa hay is quite efficient for milk protein building   From the fact that alfalfa hay contains a relatively small amount of acid amide nitrogen and a much greater amount of amino-acid nitrogen, they drew the conclusion that the real nutritive nitrogen value of alfalfa hay lay in the amino-acid nitrogen   They made[17] a comparison of the relative efficiency of the ingestion of the proteins of milk, corn, and wheat grain on milk production, they found milk protein most effective and wheat grain least effective   During the negative nitrogen balance which followed corn or wheat protein ingestion they observed enhanced tissue autolysis The production of milk remained the same both in volume and concentration for a short period at the expense of catabolized tissue

McCollum, Simmonds, and Pitz[18] found that young rats were able to grow normally upon a ration containing wheat, casein, dextrin, butter fat, and salt mixture   Females gave birth to litters of young, but the mothers failed to rear young on this diet   The lack of suitable milk production by the mother was due to the shortage of the water-soluble accessory factor in the milk   These results are the experimental proof of their earlier statement that the unidentified fat-soluble and water-soluble accessory substances of the diet "pass into the milk only as they are present in the diet of the mother, and that milks may vary in their growth-promoting power when the diets of the lactating animals differ widely in their satisfactoriness for the growth of young "[19]

Daniels and Nichols,[20] working with the soy bean rations, came to the conclusion that very young rats required a greater amount of fat-soluble accessory factor than adults to pass the early growing period successfully

[15] Osborne, T. B., and Mendel, L B , *J Biol Chem* , 1913, xv, 311

[16] Hart, E B , and Humphrey, G C , *J Biol Chem* , 1914, xix, 127

[17] Hart, E B , and Humphrey, G C , *J Biol Chem* , 1915, xxi, 239

[18] McCollum, E V , Simmonds, N , and Pitz, W , *J Biol Chem* , 1916–17, xxviii, 211

[19] McCollum, E V , Simmonds, N , and Pitz, W , *J Biol Chem* , 1916, xxvii, 33

[20] Daniels, A. L , and Nichols, N B , *J. Biol Chem* , 1917, xxxii, 91

Loeb and Northrop[21] have been able to show that the imago of the fruit fly (*Drosophila*) can live on "glucose-agar" alone, while the larvæ cannot grow on "glucose-agar" unless yeast is added   The larvæ needed an adequate diet for body building while the imago, the full grown organism, appeared to be in no need of such growth-inducing substances as are present in yeast

Stepp[22] has pointed out that the substance in cow's milk, which in minute quantities suffices to induce normal growth of young mice when added to an inadequate diet, is not fat, cholesterin, lecithin, or salts

Meigs and Marsh[23] obtained two unknown substances from cow's milk, one alcohol-soluble and the other ether-soluble, which are claimed to be important constituents of diets   The former substance contained 13 8 per cent of nitrogen and its ash showed the presence of phosphorus, while the latter contained no nitrogen, but contained a considerable amount of sulfur   The amount of these substances diminished with the progress of lactation

Methods and Material Employed.

The albino rats used by us were raised in our own laboratory. They were fed with wheat bread soaked in whole milk, fresh carrots. and occasionally a small amount of fresh meat (beef). This normal diet was designated as Ration N.

The animals were from 90 to 300 days old   This includes the period of rapid growth and the attainment of maturity   Also the most successful reproductions occur in this period.   Pregnant animals on a normal diet were placed in separate metal cages, having sawdust- and newspaper-sprinkled floors, as soon as their condition was discovered and were put on the special diets which were continued until the end of the experimental period.   In choosing the pregnant females among normally fed animals, care was taken to select only those in good health.   A large number of pregnant rats were fed with the normal diet at the same time that the experimental diets were being investigated.

The animals were weighed together upon the day of birth (less than 12 hours after birth) and every alternate day   The size of the litter as well as the physical condition and body weight of the mother at the birth of the young and during the experi--

[21] Loeb, J , and Northrop, J H , *J Biol Chem* , 1917, xxxii, 103

[22] Stepp, W , *Z Biol* , 1911-12, lvii, 135

[23] Meigs, E B , and Marsh, H L , *J Biol. Chem* , 1913-14, xvi, 147.

mental period were recorded. The sex of the new-born animals was determined by the method devised by Jackson [24]

It is a well known fact that generally male albino rats weigh more than females throughout life   King[25] has found in 85 litters examined that the average weight at birth of the male albino rats is 4 54 gm , and that of the females is 4 27 gm , while Jackson[24] reported 5 13 gm for males and 4 89 gm. for females in 63 and 66 animals respectively.   The different factors which might influence the body weight of the albino rats at birth are summarized by King as follows (a) The age of the mother; (b) the physical condition of the mother, (c) the body weight of the mother; (d) the size of the litter, (e) the position of the litter in the litter series; and (f) the length of the gestation period [25]

King's interesting graphic comparison[26] shows that during the first 60 days the growth curve of female albino rats runs very closely to the growth curve of the males, but then the curves begin to separate rapidly, i e , males surpassing females in body weight

We have not attempted to weigh young males and females separately during the first 30 days for two reasons   first, there is no object in the determination of the body weights at definite ages, second, we are interested only in seeing what difference there is in the body weight and general condition of the young when the mother's diet is changed to the experimental diet.

The relative effective value of foods was determined by the change in the body weights of young rats at seven selected ages, namely, at birth, and on the 5th, 10th, 15th, 20th, 25th, and 30th days, and comparing these weights with those of young whose mothers were fed on a normal diet

The following rations were employed in this investigation and the methods of preparation for the individual food substances are discussed in detail.

[24] Jackson, C M , *Biol. Bull* , 1912, xxiii, 171

[25] King, H D , *Anat Rec* , 1915, ix, 213

[26] King, H D , *Anat. Rec* , 1915, ix, 751

Ration N

.   Bread
     Carrots
     Milk

Ration Y

	per cent
Bananas	83 5
Casein	16 0
Yeast .	0 5

Ration M

	per cent
Bananas	83 5
Casein	16 00
Yeast .	0 5
Milk .	10 0 cc each animal

Ration P

	per cent
Bananas .	. 83 0
Casein	16 0
Yeast	. 0 5
Protein-free milk .	. 0 5

Ration S

	per cent
Bananas	83 22
Casein	16 00
Yeast	0 50
Salt mixture	0 28

Ration N S

	per cent
Bananas .	83 22
Casein	16 00
Yeast	0 50
Natural salt mixture	0 28

Ration L

	per cent
Bananas	83 00
Casein .	16 00
Yeast	0 50
Lactose	0 50

Bananas —In a recent article Hess and Unger[27] have shown that fresh young vegetables possess a much greater amount of antiscorbutic and growth-promoting substances than old vegetables.

[27] Hess, A. F , and Unger, L J , *J Biol Chem* , 1919, xxxviii, 293.

Abderhalden and Lampé[28] and Funk[29] showed that cooked polished rice took much longer to produce polyneuritis in pigeons than uncooked polished rice   They suggested that the beneficial action of the cooked rice was due partly to the intake of relatively smaller amounts of carbohydrate by the birds

In a preliminary communication,[1] we have reported that a banana diet maintained the life of young albino rats much longer than when fed on carrots as the sole food   We believed that the difference in the nutrition of young animals when fed upon these closely allied forms of foods is due partly to a difference in the degree of digestibility   Another example of how easily digestible foods influence the maintenance of animal life is seen in the fact that rats can live very much longer on the cooked white potato (Irish) than on uncooked potato. Detailed investigation on the nutritive value of potato will be given in a later paper   Throughout the experiments we used only the edible portion of well ripened, golden yellow bananas.

Casein.—McCollum and Davis have purified casein without any application of high temperature since they learned that the prolonged heating even at temperatures of 90–100°C   causes deterioration of the nutritive properties of milk. The growth curves showed the absence of unknown growth-promoting substances in their purified casein [30]   They have observed that heating casein in a moist condition for 1 hour in an autoclave at 15 pounds pressure destroys its biological value as a complete protein [31]   This particular point is true when a ration contained 5 per cent casein,[32] but when a ration contained 10 per cent casein,[33] the difference of the nutritive value in the heated and unheated casein is not clearly shown

Funk and Macallum,[34] in order to free commercial casein from the traces of unknown accessory substances, extracted it by

[28] Abderhalden, E , and Lampé, A E , *Z ges. exp Med* , 1913, i, 296.

[29] Funk, C , *Z physiol Chem* , 1914, lxxxix, 373.

[30] McCollum, E V , and Davis, M , *J Biol Chem* , 1915, xxiii, 231

[31] McCollum, E V , and Davis, M , *J Biol Chem* , 1915, xxiii, 247

[32] McCollum and Davis, [31] Chart 5

[33] McCollum and Davis,[31] Chart 6

[34] Funk, C , and Macallum, A. B , *Z physiol. Chem* , 1914, xcii, 13, *J Biol. Chem* , 1916, xxvii, 51

refluxing for 6 hours with boiling 95 per cent alcohol. They found that the process did not alter the nutritive properties of the protein

Drummond[35] has extracted the dried caseinogen with two changes of alcohol for 6 hours at 60°C., and then with ether for 6 hours   This method of purification did not make it unsuitable for the rats   However, he has observed in two cases that when the caseinogen was extracted with hot alcohol in a slightly moist condition, there was some chemical change and it lost its protein value

The casein we employed was prepared from commercial, washed casein by boiling for 2 hours with 95 per cent ethyl alcohol   It was filtered after standing over night at room temperature and the casein washed well with fresh alcohol and then allowed to dry in the air.   A former experiment[36] showed clearly that our purified casein was free from unidentified accessory factors, it was not toxic, and it possessed full biological value as a complete protein

Yeast—The shortage of water-soluble accessory substance in bananas was supplied from yeast.[37,38]   Fresh yeast[39] was filtered, pressed, dried in the air at room temperature, and was then well ground   During drying, mold has generally grown on the surface of the yeast.

Protein-Free Milk.—The fact that young white rats have failed to grow upon the isolated food substances, but rapid recovery of health and growth have followed when 28 per cent of protein-free milk has replaced the inorganic elements and a part of the carbohydrate in food, led Osborne and Mendel to conclude that their natural protein-free milk   contains unidentified water-soluble accessory substances [40]   Later Osborne and Mendel[38]

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[35] Drummond, J. C , *Biochem J* , 1916, x, 89

[36] Sugiura, K , and Benedict, S R , *J Biol Chem* , 1919, xxxix, 421, Experiment 1

[37] Funk, C , and Macallum, A B , *J. Biol Chem* , 1915, xxiii, 413

[38] Osborne, T. B , and Mendel, L B , *J Biol Chem* , 1917, xxxi, 149

[39] The yeast was obtained from the Lion Brewery, New York City.

[40] Osborne, T. B., and Mendel, L B , *Carnegie Institution of Washington, Publication No 156*, 1911, pt 2, *Z physiol Chem* , 1912, lxxx, 356; *J Biol Chem* , 1912, xii, 473; 1913, xv, 311; 1915, xx, 351; 1916, xxvi, 1

have shown that the protein-free milk appeared to contain a new unidentified accessory substance which is not present in yeast.

We have prepared natural protein-free milk from fresh skimmed milk[41] according to the procedure used by Osborne and Mendel [42] Different chemical analyses show that the contents of our preparations are nearly the same as those found by Osborne and Mendel.

Salt Mixtures —The importance of the individual inorganic salts in the rôle of nutrition has been clearly shown and discussed [42, 43] Our artificial salt mixture was prepared according to Osborne and Mendel [44] The natural salt mixture was prepared by igniting our protein-free milk until entirely free from carbon The inorganic residue gave 14 3 per cent of the original material.

Lactose.—Hopkins and Neville,[45] Sweet, Corson-White, and Saxon,[46] McCollum and Davis,[47] and Drummond[35] have clearly shown that the lactose, prepared from milk, may contain traces of impurities which act as a growth-promoting substance, and such substances can be removed by means of purification with 95 per cent alcohol.

We have purified lactose (Merck) by means of recrystallization from ethyl alcohol The crystalline lactose was dried in a vacuum desiccator over sulfuric acid

[41] The milk was obtained from the Walker-Gordon Laboratory Company, New York City

[42] Osborne, T B , and Mendel, L B , *Carnegie Institution of Washington, Publication No 156*, 1911, pt 2

[43] Rohmann, F , *Allg med Central -Z* , 1908, lxxvii, 129 McCollum, E V , *Am J Physiol* , 1909–10, xxv, 120 Evvard, J M , Dox, A W , and Guernsey, S C , *Am J Physiol* , 1914, xxxiv, 312 Hart, E B , and McCollum, E V , *J Biol Chem* , 1914, xix, 373 Hogan, A G., *J Biol Chem* , 1916, xxvii, 193 Osborne, T B , and Mendel, L B , *J Biol Chem.*, 1918, xxxiv, 131 Steenbock, H , Kent, H E , and Gross, E. G , *J Biol Chem* , 1918, xxxv, 61 Sherman, H C , Chemistry of food and nutrition, New York, 2nd edition, 1918, 234

[44] Osborne, T B , and Mendel, L B , *J Biol Chem* , 1913, xv, 317, Salt Mixture IV, minus the lactose

[45] Hopkins, F G , and Neville, A., *Biochem J* , 1913, vii, 97

[46] Sweet, J E , Corson-White, E P., and Saxon, G J , *J Biol Chem* , 1915, xxi, 309

[47] McCollum, E V , and Davis, M., *J. Biol. Chem* , 1915, xxiii, 181

Experimental Results.

Our experimental results are compiled in Tables I to VII.

From the experiments we obtained the following facts.

(1) The comparative number of young born per litter from mothers fed with different rations is summarized in Table VIII. (2) The average number of days when the young from mothers fed on the different diets opened their eyes was noted and found to be 15 6 for Ration N, 16.0 for Ration Y, 16 0 for Ration M, 15 6 for Ration P, and 15 9 for Ration L. (3) The total per cents of young rats which were born under the following diets and which have thrived during the experimental periods of 30 days were found to be with the normal diet, 71; with Ration Y, 20; with Ration M, 87, with Ration P, 71; and with Ration L, 26. (4) The addition of 0 5 per cent of protein-free milk to the mother's diet, which consisted of bananas, 83 0, casein, 16 0, and yeast, 0 5 per cent, not only maintained her body weight throughout the lactation period, but also gave excellent milk supply both in quantity and quality (Table IV). (5) The amount of the food, Ration P, eaten by the mother during the lactation period increased as lactation progressed (6) The addition of 0 28 per cent of salt mixture, either artificial or natural, had no beneficial action upon the banana-casein-yeast diet (Tables V and VI) (7) The addition of 0.5 per cent of purified lactose to the banana-casein-yeast diet increased slightly its nutritive value. A small number of young were reared by the mothers on this diet, but their body weights were very much below normal (Table VII). (8) Table IX shows more clearly the relative effective value of foods The weights are the average of the rats which survived during the experimental periods. These figures are compiled from Tables I to VII.

TABLE I

Ration N —Bread, Carrots, Milk

Mother		No of litter		Average body weight of males and females together							Remarks
No	Body weight	Males	Females	Birth	5th day	10th day	15th day	20th day	25th day	30th day.	
	gm			gm	gm	gm	gm	gm	gm	gm	
60	147	4	6	3 81	5 66	7 45	9 83	13 30	17 27	22 08	1 ♀ died on the 17th, 1 ♂ on the 18th, 1 ♀ on the 21st day, rest grew, somewhat undersized.
60	160	5	5	3 90	*			.			1 ♂ died shortly after birth, rest died on the 2nd day
282	130	4	5	4 93	7 23	10 72	13 93	17 72	21 50	24 18	Animals grew well, general condition excellent
283	178	4	3	5 21	9 70	13 59	†				1 ♀ died shortly after birth; rest killed and eaten on the 12th day
294	204	2	3	5 62	10 17	16 76	24 15	32 92	41 74	47 04	Animals grew well, general condition excellent
219	138	7	1	5 00	7 41	10 12	11 55	13 26	17 52	21 80	" " somewhat undersized
104	178	7	1	4 85	8 05	11 27	14 79	18 18	21 96	24 05	" well, general condition excellent
374	198	9	3	5 03	8 04	10 91	13 26	†			Killed and eaten on the 17th day
376	144	4	5	4 35	7 31	12 26	16 33	20 29	25 01	31 77	1 ♂ and 1 ♀ eaten on the 2nd day, rest grew well, general condition excellent.
377	163	5	5	4 74	7 26	11 12	14 94	19 91	24 45	30 89	Animals grew well, general condition excellent
431	153	5	3	5 09	7 74	10 51	13 63	18 05	22 90	28 30	" " "
445	106	3	1	5 15	8 39	12 92	18 25	25 40	32 90	41 00	" " "
249	167	7	3	4 34	5 77	8 19	11 18	14 59	17 99	22 09	" " somewhat undersized
481	225	4	2	5 98	11 04	17 90	25 20	32 98	43 34	51 84	" well, general condition excellent

* Died

† Animals were killed and eaten by mother

TABLE II.

Ration Y —Bananas 83 5, Casein 16 0, Yeast 0 5 Per Cent.

Mother		No of litter		Average body weight of males and females together							Remarks
No	Body weight	Males	Fe-males	Birth	5th day	10th day	15th day	20th day	25th day	30th day	
	gm			gm	gm	gm	gm.	gm	gm	gm	
65	126	3	2	4 33	*						2 ♂ died on the 1st day; 1 ♂ and 1 ♀ on the 3rd day. 1 ♀ on the 4th day
77	117	2	0	5 01	*						All died on the 1st day
65	117	4	5	2 93	*						2 ♀ died on the 1st day, rest died on the 2nd day
193†	128	3	3	4 45	6 00	6 02	*				2 ♀ and 1 ♂ died on the 9th, on the 11th 1 ♂, on the 12th 1 ♂ and 1 ♀
219†	135	5	7	3 73	6 13	8 43	10 48	11 33	12 62	16 53	1 ♂ died on the 26th day, and 1 ♂ eaten, rest poor and development retarded.
220†	103	5	4	4 16	5 13	7 28	9 40	11 03	‡		3 ♂ and 2 ♀ died on the 21st day, and 1 ♂ and 2 ♀ eaten, on the 22nd day 1 ♂ died
62†	125	3	4	4 39	4 00	‡		.			1 ♂ died on the 2nd day, 1 ♂ died and 1 ♀ eaten on the 4th; on the 6th 3 ♀ died and 1 ♂ eaten

* Died

† Animals were on normal diet when they became pregnant

‡ Animals were killed and eaten by mother

TABLE III

Ration M.—Bananas 83.5, Casein 16.0, Yeast 0.5 Per Cent and Milk 10 Cc for Each Animal

Mother		No of litter		Average body weight of males and females together							Remarks
No	Body weight	Males	Females	Birth	5th day	10th day	15th day	20th day	25th day	30th day	
	gm			gm.	gm	gm	gm	gm	gm	gm	
49*	132	5	4	3 98	7 00	8 86	13 38	17 18	21 78	26 13	2 ♂ and 2 ♀ died on the 2nd day, rest grew well, general condition excellent.
91*	124	4	5	4 02	6 72	9 57	11 63	12 82	15 80	19 15	Animals grew, somewhat undersized
283†	151	5	2	5 05	7 44	13 14	17 43	22 00	26 95	32 17	" " well; general condition excellent.
219†	144	3	3	4 27	6 60	9 86	12 27	17 91	22 09	28 14	" " " "

* Animals were on Ration Y when they became pregnant.
† Animals were on normal diet when they became pregnant

TABLE IV

Ration P —Bananas 83 0, Casein 16 0, Yeast 0 5, Protein-Free Milk 0 5 Per Cent

| Mother | | No of litter | | Average body weight of males and females together | | | | | | | Remarks |
No	Body weight	Males	Females	Birth	5th day	10th day	15th day	20th day	25th day	30th day	
	gm			gm	gm	gm	gm	gm	gm	gm	
65†	123	3	2	4 80	6 64	9 94	13 67	16 97	23 31	32 89	Animals grew well, general condition excellent
210	124	1	5	4 35	7 35	11 55	15 50	19 72	26 96	32 91	" " "
222*	130	4	3	4 22	†						All died on the 1st day
49*	139	4	3	4 29	6 77	11 52	15 26	18 54	27 12	36 17	Animals grew well, general condition excellent.
104*	183	4	7	5 15	8 35	11 92	14 32	15 78	20 31	24 20	General condition poor and development retarded
196*	143	2	3	4 91	10 67	16 14	20 64	27 16	38 11	45 51	Animals grew well, general condition excellent
248*	152	4	3	5 01	8 83	12 46	‡				1 ♂ died on the 2nd day; during 11th day all eaten

* Animals were on normal diet when they became pregnant

† Died

‡ Animals were killed and eaten by mother

TABLE V.

Ration S.—Bananas 83 22, Casein 16 00, Yeast 0 50, Salt Mixture 0 28 Per Cent

Mother		No of litter		Average body weight of males and females together							Remarks
No	Body weight	Males	Females	Birth	5th day	10th day	15th day	20th day	25th day	30th day	
	gm			gm	gm	gm	gm	gm	gm	gm	
214*	117	4	3	4 06	†						1 ♂ and 1 ♀ died shortly after birth, rest died on the 2nd day
217*	108	4	4	3 53	†						3 ♂ and 2 ♀ died on the 1st day, rest died on the 2nd day
282*	142	2	3	4 88	5 24	‡					All eaten on the 6th day

* Animals were on normal diet when they became pregnant

† Died

‡ Animals were killed and eaten by mother

TABLE VI

Ration N S.—Bananas 83 22, Casein 16 00, Yeast 0 50, Natural Salt Mixture 0 28 Per Cent

Mother		No of litter		Average body weight of males and females together							Remarks
No	Body weight	Male	Female	Birth	5th day	10th day	15th day	20th day	25th day	30th day	
	gm			gm	gm	gm	gm	gm	gm	gm	
376*	198	5	3	4 07	5 28	†					1 ♂ and 1 ♀ died on the 3rd, on the 4th 2 ♂ and 1 ♀, and on the 6th the rest died
374*	162	2	4	4 32	‡						Killed and eaten on the 2nd day

* Animals were on normal diet when they became pregnant.

† Died

‡ Animals were killed and eaten by mother.

TABLE VII

Ration L.—Bananas 83 0, Casein 16 0, Yeast 0 5, Lactose 0.5 Per Cent

Mother		No of litter		Average body weight of males and females together							Remarks
No. *	Body weight	Male	Female	Birth	5th day	10th day	15th day	20th day.	25th day	30th day	
	gm			gm.	gm	gm.	gm	gm	gm	gm	
60	157	5	3	4 22	†						Eaten on the 2nd day
234	111	2	3	4 64	7 00	10 04	13 71	16 25	19 24	20 52	Animals grew, somewhat undersized
217	121	2	4	5 14	4 70	†					1 ♀ died on the 5th; on the 6th day 2 ♂ and 2 ♀ died and 1 ♀ eaten
219	142	?		†							Killed and eaten by mother
280	131	6	2	3 87	4 62	†					2 ♂ died on the 2nd day; on the 3rd 2 ♂ and 1 ♀ eaten, on the 6th 1 ♂ and 1 ♀ eaten; rest died on the 7th day.
220	134	5	2	4 13	5 76	7 43	8 89	9 97	11 86	13 63	General condition poor; development retarded
217	142	3	3	5 00	6 00	†					All young died during the 8th day
378	127	5	2	5 32	7 33	8 58	7 58	8 35	†		2 ♂ and 1 ♀ died on the 15th day, on the 16th 1 ♀; on the 20th day 3 ♂.
385	151	2	4	5 02	7 03	10 50	14 44	19 18	23 62	28 09	Animals grew well; general condition excellent
411	119	5	4	4 21	7 04	9 79	16 68	19 58	23 13	27 90	1 ♀ eaten on the 2nd day, on the 3rd 1 ♂ eaten; on the 9th 1 ♀, on the 11th day 1 ♂ died; rest grew well, general condition excellent
341	113	3	3	3 93	5 37	7 03	6 75	6 23	†		1 ♂ died shortly after birth; on the 2nd 1 ♀, 1 ♂ on the 17th; 1 ♀ on the 18th, rest died on the 20th day
374	155	2	3	4 02	†						Mother ate all on the 2nd day.

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										Remarks
473	152	2	6	4 78	6 90	7 25	8 30	8 65	9 79	10 60	1 ♂ eaten on the 2nd day, on the 13th 1 ♀ died, on the 24th 2 ♀ died, on the 28th 1 ♀; on the 29th 2 ♀ eaten, rest, general condition poor, development retarded
376	190	5	3	4 81	7 66	12 18	15 26	17 48	19 64	22 66	2 ♂ eaten on the 2nd day, on the 6th day 1 ♂ eaten, rest grew, somewhat undersized.
249	130	6	4	3 30	†						5 eaten on the 1st day; on the 2nd 1 ♂ and 1 ♀, on the 3rd 1 ♂ and 1 ♀, on the 4th 1 ♂
276	115	6	0	4 03	4 13	‡					All young died on 7th day
341	118	4	3	4 57	4 38	‡					" " " 8th "
385	116	3	2	4 59	7 12	11 82	16 65	22 16	26 24	29 82	1 ♂ eaten shortly after birth; on 2nd day 1 ♂ and 2 ♀ eaten; rest grew well, general condition excellent.

* All animals were on normal diet when they became pregnant.
† Animals were killed and eaten by mother.
‡ Died.

TABLE VIII

Mother's ration	No of litters examined	Average no of young per litter
N.	14	8 3
Y.	7	7 1
M	4	7 8
P	7	6 9
S.	3	6 7
N S.	2	7 0
L	18	6 5

TABLE IX

Day of determination	Weight of rats on experimental diets						
	Ration N	Ration Y	Ration M	Ration P	Ration S	Ration N S	Ration L.
	gm	gm	gm	gm	gm	gm	gm
Birth	4 86	4 14	4 33	4 68	4 16	4 20	4 45
5th.	7 98	5 32	6 94	8 10	5 24	5 28	6 07
10th	11 82	7 24	10 36	12 26	+	*	9 40
15th	15 59	9 94	13 68	15 88			12 03
20th	20 60	11 18	17 48	19 63			14 21
25th	26 05	12 62	21 66	27 16			19 07
30th	31 37	16 53	26 40	34 34			21 89

* Died

DISCUSSION.

Our results show that whole milk, or protein-free milk is effective in making a banana-casein-yeast diet complete for milk production

According to Osborne and Mendel,[11] their protein-free milk contains 0 48 per cent of non-protein nitrogen, and 0.28 per cent of protein nitrogen.   They have stated that the amount of milk protein in the protein-free milk was not the cause of inducing the growth of the retarded animals   On the other hand, McCollum and Davis[48] argue that the nitrogen of the protein-free milk is equivalent to milk protein nitrogen as a nutrient for young rats.   Osborne and Mendel[38] answer the criticism of McCollum

48 McCollum, E V , and Davis, M , *J. Biol. Chem* , 1915, xx, 641

and Davis by showing very remarkably different chemical properties possessed by the protein-free milk and yeast.   They say.

"On a ration of purified casein, 'artificial protein-free milk,' starch, laid, butter fat, and 1 5 per cent of dried yeast, rats of both sexes have grown from about 50 gm body weight to maturity, and have even produced young   .   Adult rats have been maintained for more than 300 days   For some as yet unknown reason the majority of the rats grew normally when the protein used was casein, whereas they have usually failed when it was edestin, and almost invariably when lactalbumin, cotton seed globulin, cotton seed proteins, or squash seed globulin was fed   This result surprised us because all of these proteins[49] had earlier led to normal growth when used in rations containing natural 'protein-free milk.' "

From these experiments, Osborne and Mendel make the suggestion that the unknown nitrogenous constituents in the protein-free milk may supply the unrecognized deficient substances in these proteins   Assuming Osborne and Mendel's figures to be correct, we added only 0 0088 per cent of milk protein from the protein-free milk   We believe this amount does not exercise any influential effect upon the nutritive efficiency of the dietary.

Interesting investigation on the nature of lactalbumin as a complete protein has been made by Emmett and Luros [50]   They concluded, from the fact that lactose supplemented lactalbumin, that the former either acted as a buffer to overcome the toxicity present in the diet, or it adsorbed a new water-soluble growth-promoting substance

In a recent paper, Kennedy[51] has reported that protein-free milk contains "either unprecipitated protein, or peptids of considerable size "

CONCLUSIONS

1. A diet consisting of bananas, 83 0 per cent, casein, 16 0 per cent, yeast, 0.5 per cent, and protein-free milk, 0 5 per cent is an adequate diet for the growth, maintenance, reproduction, and perfect milk production of the albino rats.

[49] Osborne, T B , and Mendel, L B , *J. Biol Chem.*, 1912, xii, 473, 1915, xx, 351, 1916, xxvi, 1

[50] Emmett, A. D., and Luros, G O , *J. Biol Chem* , 1919, xxxviii, 147.

[51] Kennedy, C., *J. Am Chem Soc.*, 1919, xli, 388

2. Protein-free milk contains a substance which is needed for suitable milk production by the mother.

3. This substance appears not to be associated with purified milk-sugar or inorganic constituents of milk.

4. This peculiarity possessed by the protein-free milk indicates that it contains a new accessory substance which is lacking in yeast.

5. Our experiments suggest that a combination of bananas and milk, in proper proportion, constitutes a complete food.

FURTHER CONTRIBUTIONS TO THE PHYSIOLOGY OF PHOSPHORUS AND CALCIUM METABOLISM OF DAIRY COWS.*

By EDWARD B. MEIGS, N R. BLATHERWICK, and C A. CARY.

With the Collaboration of T. E Woodward

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Recent work has made it seem probable that the amount of calcium and phosphorus contained in the rations of dairy cows is a matter of great practical importance   In a comprehensive series of experiments on liberally milking cows, Forbes and co-workers[1] have found that the calcium balances were always negative and the phosphorus balances usually so, and this in spite of the fact that the rations were liberal and, in many cases, contained more calcium and phosphorus than is ordinarily fed even to high producing cows.

In view of these and other results with the same general tendency, it seems desirable that calcium and phosphorus metabolism in dairy cows be intensively studied, and we have recently carried out a series of experiments on cows in which the calcium, phosphorus, and nitrogen balances were followed.

Objects of the Experiments.

Our experiments were planned to throw light on a number of points.   It has been the custom at the Government Farm at Beltsville, Md., as it probably is on most dairy farms, to feed cows according to their milk yield.   As the milk yield decreases

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[1] Forbes, E B , Beegle, F M , Fritz, C M , Morgan, L E , and Rhue, S. N., *Ohio Agric Exp Station, Bull 295*, 1916; *ibid , Bull 308*, 1917 Forbes, E B , Halverson, J O , and Morgan, L E , *ibid , Bull. 330*, 1918.

with the progress of lactation, the feed—particularly the grain—is reduced, and reaches its lowest point when the cow becomes altogether dry, generally only a few weeks before the next calf is born   The routine ration fed to the dry cows at Beltsville is shown in Table III, as the ration fed to Cow 51 from February 17 to March 10, 1919.   It contains sufficient protein, fat, and carbohydrate to provide liberally for the maintenance of the cow and the growth of the unborn calf, as calculated from the figures given in the standard text-books.

In most dairy rations, the grain contains a large proportion of the phosphorus, and the ration in question is no exception to this rule (Table V)   As it is the grain which is chiefly reduced in the rations of the dry cows, it becomes an interesting question whether these cows receive sufficient phosphorus to provide for the optimum milk yield in the next lactation.

We have recently reported experiments in which the concentration of phosphorus in the blood plasma of dry, pregnant cows has been followed.[2]   Though the cows were fed a much higher grain ration than the one in question, there was nevertheless a decided tendency for the concentration of phosphorus in the blood plasma to fall off toward the end of pregnancy   In other unpublished experiments, we have found that this tendency is more marked when the grain ration is lower.

We are engaged at present in carrying out experiments to determine whether the ration described above can be improved from the practical standpoint by adding sodium phosphate to it, and by feeding it in a manner which we judged would facilitate the absorption of phosphorus   There is considerable evidence to show that the absorption of phosphorus from the intestinal tract may be hindered by the simultaneous presence of calcium compounds.[3,4]   We have, therefore, attempted to separate the calcium

[2] Meigs, E. B , Blatherwick, N R., and Cary, C A , J Biol Chem , 1919, xxxvii, 1.   The experiments in question are referred to on pp 45 and 46

[3] Hammarsten, O , A text-book of physiological chemistry, translated by Mandel, J. A , New York and London, 7th edition, 1915, 762   Forbes, E B , with collaborators, Ohio Agric Exp. Station, Techn Bull 6, 1915, 66

[4] Bertram, J , Z. Biol , 1878, xiv, 335.

and phosphorus compounds in the intestinal tract by feeding the grain and hay of the rations on alternate days. This method of feeding is shown in Table III as the ration fed to Cow 63 from February 17 to March 10.

The milk yield of cows fed, for several weeks before calving, in this manner has been compared with that of control animals fed like Cow 51, February 17 to March 10 (Table III)   So far, about a dozen experiments and controls have been carried through altogether, and the results indicate that the cows on the experimental feeding gain weight and improve in condition at least as satisfactorily as the controls and that their milk yield in the next succeeding lactation is decidedly larger

We do not consider the results sufficiently numerous as yet to be final, and we are carrying out further experiments of the same sort.   But, in the meantime, we wished to study the effects of the experimental feeding on calcium and phosphorus assimilation, and the experiments to be reported in this article have been planned with that end in view

This made it possible to study the effects of feeding phosphate on the urinary and fecal excretion of phosphorus   We have previously shown that feeding phosphate tends to increase the concentration of phosphorus in the blood [5]   It seemed worth while, therefore, to take a certain number of blood samples during the course of the balance experiments and to study the triple relationship between phosphorus in the rations, in the blood, and in the urine

General Plan of Experiments.

Two healthy, grade Holstein cows were selected, each of which had been dry for about a month.   Each had been fed for some time previous to the beginning of the experiment on the same ration that she was to have during the first part of the experiment   Both were in the last third of pregnancy   One, No. 51, was due to calve on April 17, 1919, 59 days after the beginning of the experiment, the other, No 63, on May 4, 1919, 76 days after the beginning of the experiment.   Their weights are given in Table I.

[5] Meigs, E B , Blatherwick, N R , and Cary, C A , *J Biol. Chem* , 1919, xxxvii, 45

On February 17, 1919, the cows were brought into the stalls in which the experiment was to be carried out. These stalls, in the basement of the laboratory building, were built of wood. Their construction was in general similar to that to which the cows were accustomed. They were provided with mangers so constructed as to prevent effectively the scattering of the rations, and with beds made of several layers of sacking. The cows were held in stanchions like those to which they were accustomed. The temperature in the experiment room ranged from 10–18°C.

The urine and feces were collected in essentially the same manner as in Forbes'[1] experiments. Three men were employed, each of whom remained behind the cows for 8 hours out of the 24, and were provided with shovels and large, galvanized iron dippers in which to catch the feces and urine, respectively, before they reached the ground. In addition, a long, galvanized iron pan was placed behind each cow to catch any of the excreta that might accidentally fall The feces caught in the shovels were transferred to covered, galvanized iron garbage cans, and the urine caught in the dippers was transferred to glass carboys. The excreta which fell accidentally were placed in separate receptacles and weighed. The total amount, however, throughout the course of the experiments was so small that no conceivable departure of its composition from the average could have had any perceptible effect on the results It was therefore simply brought into the calculation as a part of the daily amount of excreta voided, but was not separately analyzed.

The experimental day began and ended at 10 a.m. At this time the urine and feces collected in the previous 24 hours were weighed and aliquot samples of each were set aside. As an aliquot from the feces we took $\frac{1}{60}$ of the total amount voided in the 24 hours and added a small amount of chloroform as a preservative From the urine we took two aliquots in each case, one for the nitrogen determination, and the other for the calcium and phosphorus determinations The first of these amounted to 20 cc per pound of urine voided, and was preserved with a small amount of chloroform The second amounted to 100 cc. per pound of urine voided. To this quantity enough strong HCl was added to make the resulting mixture distinctly acid to litmus after all the CO_2 had been driven off. The amount of HCl added was measured and recorded, and a small amount of chloroform was added.

The experimental period lasted in each case for 4 days. The aliquots obtained during this period were thoroughly mixed, and in this way representative samples were obtained for analysis. The results, however, are given as the daily average for each of the 4 day periods.

On February 17, 1919, a blood sample was obtained from each cow at about 8 a m , the cows were brought into the experimental stalls about 9 a.m , and their urine was collected for the next 18 hours. From February 18 until March 2, 1919, inclusive, the urine and feces were not collected, but otherwise the cows were kept under the same routine as during the collection periods. The rations given to each are shown in Table III.

The balance experiment began on March 3. About 8 a.m. on this date a second blood sample was obtained from each cow, and at 10 a.m. the collection of the urine and feces was begun. This was continued until 10 a.m on March 11, the intervening 8 days being divided into two 4 day collection periods as described above. On the morning of March 11, a third blood sample was obtained from each cow, and the rations were reversed as shown in Table III. The period from March 11 to 20, inclusive, served as an intermediate period to accustom the cows to the new rations. The excreta were not collected

On the morning of March 21, it was evident that No. 51 was going to abort, and she had to be discarded as a subject for the experiment The abortion took place that evening. The calf was born dead, but it weighed about 30 kilos and was well formed for a calf born 4 weeks too early. A few weeks later No. 51 was tested for contagious abortion and reacted positively.

It was decided to carry the experiment on with No 63 as originally planned, and to substitute for No 51 another cow, No. 66, which had for 88 days previously been receiving the same alternated rations that were supplied to No. 51 from March 11 to 20. This cow was a grade Guernsey, and was neither pregnant nor milking, but it was thought that some further data could be obtained from her in regard to the effects of phosphate feeding on the concentration of phosphorus in the blood and the elimination of phosphorus and calcium in the urine.

At 7 30 on the morning of March 21, therefore, blood samples were obtained from Nos 63 and 66 and at 10 a.m the collection

of urine and feces was started It was continued as before for two 4 day periods. At the end of the second one, blood samples were again taken and the experiment was concluded.

No 63 gave birth to a normal calf, which weighed 31.4 kilos, on the morning of April 25, 1919

The cows were, of course, closely watched for any abnormalities of appetite or general behavior. No 51 showed no outward sign of disturbance by the experimental routine at any time, except that she drank less water during the collection periods than during the intermediate period (Table II).

No 63, on the other hand, gave signs of being disturbed by various parts of the procedure. During the preliminary period (February 17 to March 2), she refused a part of her ration, although she had eaten it well for the entire previous 2 weeks in the general barn; her silage had to be reduced She consumed the reduced ration for a day or two, but began to refuse some of it again as soon as the collection of urine and feces began During the first 60 hours of the first collection period, she did not lie down in her stall, probably on account of abrasions which had developed on her hips and shoulders, and toward the end of this 60 hours she became obviously extremely nervous and uncomfortable The difficulty was overcome by providing her with sacks filled with straw for a bed.[6] Like No 51, she drank less water in the collection periods than in the intermediate period. She also consistently refused some of her hay and silage in the collection periods in spite of the fact that her ration had been cut down. During the intermediate period, March 11 to 20, she refused only very small portions of her feed (Table IV).

No. 66, March 21 to 28, suffered, of course, from the double disturbance of new surroundings and collection of urine and feces Her appetite was not much affected. She ate her rations, except for the stems of the hay and the cobs in the silage, but she drank very little water, and was obviously nervous under the experimental routine She would start when anyone went near her or touched her, and would often begin the act of urination, and then discontinue it when the attendant approached her.

[6] All cows were provided with beds of this sort from the beginning of the intermediate period, Mar 11 to 20

Analytical Methods.

In undertaking our analyses of the materials fed to the cattle, we realized keenly the difficulties which might lead us into error. The errors to be expected may be summed up under two heads; those due to the fact that all the feeding materials contain a considerable and more or less inconstant percentage of water, and those due to the difficulty of obtaining representative samples for analysis—particularly from such materials as corn silage and alfalfa hay. Our preliminary experience showed us that these difficulties are very real.

They are to be avoided by grinding large samples, by thorough mixing of the ground material, and by making sufficiently numerous moisture determinations   The process of grinding often produces very considerable changes in water content, which must be adequately controlled.

We evolved a procedure which gave satisfactory results, but we do not think it necessary to give it in detail   Its detailed description would occupy a great deal of space, and the precautions are such as would occur to anyone seriously interested in the subject. We prepared enough hay and grain at the beginning to last through the experiment, but with the silage we found it advantageous to take out aliquot samples for analysis from each feed   The aliquots for each collection period were thoroughly mixed and analyzed separately from those of the other periods, and in our tables, therefore, the composition of the silage fed is given for each period separately. The composition of the various feeds is given in Table V, and the composition of the feed refused, in Table VI In a footnote to Table IV are given details concerning the manner in which the feed refused was handled.

The analytical methods used, were, in general, those described in our previous article.[5] Some modifications have, however, been introduced as indicated below.

For calcium determinations we ashed some of our samples of feces and some of our samples of hay in platinum crucibles over a free flame or in an electric furnace, instead of with nitric and sulfuric acids   We found it most convenient to determine calcium in urine directly without ashing, as suggested by McCrudden.[7]

[7] McCrudden, F. H , *J Biol. Chem.*, 1911–12, x, 199.

In some calcium determinations, also, in material ashed with nitric and sulfuric acids, we omitted the preliminary precipitation of the calcium as sulfate, our new procedure being as follows:

The acid ash is boiled with about 25 cc. of water as usual and then made up to approximately 75 cc. with water. One drop of 0.006 per cent phenolsulfonephthalein is added and 28 per cent NH_3 until the color changes through yellow to pink The mixture is cooled and 10 per cent HCl is added drop by drop until the color changes back to clear yellow; 10 cc. of 0.5 N HCl and 10 cc. of 1.75 per cent $H_2C_2O_4$ are then added; the mixture is heated to boiling; 20 cc. of saturated $(NH_4)_2C_2O_4$ solution are added; and the mixture is kept gently boiling until the precipitate becomes coarse (15 to 30 minutes) It is then cooled to 50° or below, 10 cc. of 10 per cent $NaC_2H_3O_2$ are added, and the calcium in the oxalate precipitate is determined according to the procedure given in our earlier article.

With these exceptions our analyses were carried out exactly as described in our earlier article, and, in the cases where we departed from the old procedure, we carried out parallel analyses on the same samples of material and satisfied ourselves that the two methods gave the same results We determined the moisture in our material by drying to constant weight at a temperature between 103 and 107°

We had a good deal of difficulty with the urine. Cow's urine often contains so little calcium and phosphorus that large samples must be taken if accurate results are to be obtained. To evaporate these large samples and ash the residues over a flame is very tedious and troublesome, while, if the samples are ashed with acid mixture, much difficulty is encountered on account of the high content of hippuric acid which changes to benzoic acid during the ashing After trying various procedures we found that it was most satisfactory to determine calcium without ashing as described above, and to determine phosphorus in samples which had been ashed with sulfuric and nitric acids. The aliquot samples of urine were immediately mixed with enough hydrochloric acid to drive off all the carbonate and render them distinctly acid to litmus, and this treatment brought about the precipitation of a large proportion of the hippuric acid. Calcium and phosphorus were determined after filtering this off. For the determination of nitrogen,

of course, it was necessary to set aside other aliquots not acidified and preserved only with a small amount of chloroform

The blood samples were treated and analyzed as described in our previous article, except that in some cases a change was made in the procedure for calcium, as described above

In regard to the size of the samples and the accuracy of the determinations in the case of the blood, the remarks given in our earlier article apply also to this investigation   The figures given in our tables for the composition of blood and plasma are the averages of duplicates which generally agreed with one another within 5 per cent [8]   The same remark applies also to our figures for the composition of the urine   For the feeds and feces we were able to take optimum samples, and the duplicates generally agreed more closely.

The results of our experiments are given in Tables I to XII at the end of the article

Calcium and Phosphorus Metabolism in Balance Experiments

Our results show a peculiar relation between calcium and phosphorus assimilation which seems to us to call for very careful consideration   In every period except that of March 3 to 6, in the case of No 51, phosphorus was assimilated in larger absolute quantity than calcium.   The phosphorus balances were positive throughout, while two out of the eight calcium balances were negative.

In six out of the eight periods, we were dealing with cows which were carrying calves, and at or beyond the 7th month of pregnancy. It is possible to form an approximate estimate of the rapidity with which calcium and phosphorus are assimilated by the embryo calf in the last 2 months of its intrauterine existence.   A calf, according to Forbes and Keith,[9] contains about 1.65 per cent CaO and 1.53 per cent P_2O_5 in its body, and a new-born calf weighs

[8] The difference obtained by subtracting one figure from the other was 5 per cent or less of the larger.

[9] Forbes, E. B , and Keith, M. H., *Ohio Agric Exp. Station, Techn Bull. 5*, 1914, 106.

about 35 kilos   A new-born calf, therefore, would have about 413 gm. of calcium and 234 gm of phosphorus in its body.

Forbes and Keith (page 110)[9] give the weights and the CaO and P_2O_5 contents of human embryos at various stages. As the human and bovine gestation periods are very nearly the same, the figures given may be taken as an approximate representation of the progress of things in the case of the calf   The human embryo at 7 months weighs about one-third of what it weighs at term, and its body contains about two-thirds the percentage of calcium and phosphorus that is contained in that of the new-born infant. The 7 months embryo, therefore, would contain about two-ninths of the absolute quantities of calcium and phosphorus contained in the new-born infant or calf. $\frac{2}{9}$ of 413 is approximately 92, and $\frac{2}{9}$ of 234 is 52   In the last 60 days of intrauterine life, therefore, the embryo calf must assimilate about 321 gm of calcium or 5.3 gm. a day, and about 182 gm. of phosphorus, or 3.0 gm. a day These figures are of course only rough approximations, but they are quite accurate enough for our purposes

Reference to Table X shows that the daily phosphorus assimilation of Cows 51 and 63 was sometimes above and sometimes below 3.0   The average for the six periods in which these cows were studied is 3.33, which corresponds satisfactorily with the calculated figure.

The results with regard to calcium metabolism are strikingly different   The calcium assimilation of our pregnant cows was always well below 5 3, the nearest figure to this being 3 5 for No. 51, March 3 to 6   The average daily calcium assimilation for our pregnant cows was only 1.44 gm.   This means that if the calves were growing at all as they should during the periods of the balance experiments their mothers must have been transferring considerable quantities of calcium from their own bones to those of their offspring.

One's first idea is that our rations must have been markedly deficient in calcium   We do not think that this was the case. We fed alfalfa hay, and the rations contained from 30 to 40 gm. of calcium daily   This quantity is generally considered liberal, and if our rations had been markedly deficient in calcium, the bad effects should have shown themselves in imperfectly formed calves and in reduced subsequent milk yield; because both cows were on

approximately the same rations they received during the collection periods for from 7 to 11 weeks before calving. None of these bad effects showed itself. It is true that No. 51 aborted, but there was much contagious abortion in the herd at the time, and she was shown to have the disease The premature calf was large and well formed for a calf born nearly 4 weeks ahead of time, and had certainly died only a few hours before its birth. No. 63 gave birth to a fair sized and perfectly normal calf at about the right time. Both cows have given much more milk in the lactation periods succeeding the experiments than they had ever given before.

We think that the true explanation of our results on calcium and phosphorus assimilation is quite different from that which has been suggested, and, in order to bring out certain important aspects of the subject, we wish to call attention to the proportions of calcium and phosphorus contained in the bodies of certain mammals, and to the results which have been obtained in balance experiments on the same species of mammals in the past.

The ratio $\dfrac{Ca}{P}$ contained in the whole bodies of mammals is fairly constant, and somewhat higher in mature than in new-born animals. For mature cattle and sheep this ratio is about 1.9.1; for mature pigs about 1.6.1.[10] Growing cattle and sheep must therefore, on the average, assimilate at least 1.9 parts by weight of Ca for 1 part by weight of P, growing pigs must assimilate about 1 6 parts of Ca for 1 part of P. The figures obtained in balance experiments carried out in the past deviate largely from these ratios and almost universally in the same direction.

We have made a representative study of the balance experiments carried out in the last 60 years, selecting those which included work on the calcium and phosphorus balances of healthy growing animals fed on normal or nearly normal rations [11,12,13] In these

[10] Forbes and Keith ⁹ These ratios represent absolute weights, not equivalents

[11] Lehmann, J , Landw. Ver -Stat , 1859, 1, 68   von Gohren, T , ibid , 1861, iii, 161   Soxhlet, Erste Bericht uber Arbeiten der landwirtschaftlichen-chemischen Versuchs-Station in Wien, 1870-71, 101   Weiske, H , J. Landw., 1873, xxi, 139. Hofmeister, V., Landw Ver -Stat , 1873, xvi, 126   Kohler, A., Honcamp, F , Just, M , Volhard, J , Popp, M , and Zahn

twelve researches are included the results of 136 separate experiments.   In all but eighteen of these the ratio $\dfrac{Ca\ assimilation}{P\ assimilation}$ is less than 1.9 for the cattle and sheep, and less than 1.6 for the pigs.

The unanimity with which balance experiments on growing animals show a deficiency in calcium intake as compared to phosphorus intake becomes still more impressive if the contrary cases are examined critically.   Two of these are from the work of Hofmeister.   In the same article that author gives figures for mature sheep which are plainly incorrect.   The figures for the mature and growing sheep were obtained by the same methods, and it is justifiable, therefore, to doubt the adequacy of the experimental methods   A satisfactory criticism of Hofmeister's work is given by Neumann [13]

Six of the other cases in which the calcium assimilation was not deficient occur in the work of Forbes, Beegle, Fritz, and Mensching on pigs   The pigs in question had been for 51 days on rations which contained from five to ten times as much phosphorus as calcium.   Their calcium and phosphorus assimilation was followed during this period, and the calcium assimilation was always observed to be markedly deficient with respect to the phosphorus.   At the end of the 51 days they were changed to a "meat meal" or skim milk ration with a comparatively high calcium content, and it is under these circumstances that the $\dfrac{Ca}{P}$ ratio becomes a little larger than the expected one.

Four more cases in which the calcium assimilation was not deficient with respect to the phosphorus are those of Sawyer, Baumann, and Stevens in their work on human beings   In this work, however, the phosphorus balances were negative throughout; and the results, therefore, cannot be taken as representing anything approaching average conditions in growing human beings.

O , *Landw Ver.-Stat* , 1904-05, lxi, 451.   Kohler, A., Honcamp, F., and Eisenkolbe, P , *ibid* , 1907, lxv, 349   Forbes, E B , with collaborators, *Ohio Agric Exp Station, Techn. Bull 6*, 1915.   Forbes, E. B., Beegle, F. M., Fritz, C M., and Mensching, J E , *Ohio Agric Exp. Station, Bull. 271*, 1914   Sawyer, M , Baumann, L , and Stevens, F , *J Biol. Chem* , 1918, xxxiii, 103

[12] Weiske, H., *Landw Jahrb* , 1880, ix, 205

[13] Neumann, J , *J. Landw* , 1893, xli, 343

Out of the 136 individual experiments on growing animals, therefore, the calcium intake is deficient with respect to the phosphorus in 118, and of the eighteen which do not exhibit this peculiarity, two are open to grave suspicion of experimental error, six were carried out under conditions which quite unusually favored an excessive calcium intake, and four are of such nature that they cannot be regarded as representing growth at all

Of the remaining six, two occur in the work of Weiske   Lambs were kept on a fairly uniform ration between the ages of 4 and 14 months, and 8 day balance periods were run at intervals of about a month.   If the conditions under which animals are put in balance experiments do not disturb calcium and phosphorus metabolism, this procedure ought certainly to give a representative picture of the manner in which these elements are assimilated between the ages of 4 and 14 months, in lambs.

The whole experiment falls readily into two main periods The first of these lasted a little more than 2 months, and in it the ratio $\dfrac{Ca\ assimilation}{P\ assimilation}$ was a little larger than is to be expected The second period includes the last $7\frac{1}{2}$ months of the experiment, during which the ratio $\dfrac{Ca\ assimilation}{P\ assimilation}$ was decidedly smaller than the expected one   The markedly low $\dfrac{Ca}{P}$ ratios during the last $7\frac{1}{2}$ months much more than compensate for the slightly high ones during the first $2\frac{1}{2}$ months; so that, if the figures were representative, the lambs' bodies at the end of the experiment would have contained much less calcium in proportion to phosphorus than is normal.

In view of the many chances of error to which balance experiments are subject, it seems to us remarkable that they should agree so nearly universally in any particular, and we think that the results point unmistakably to the conclusion that calcium assimilation is interfered with by the conditions to which animals have been subjected in these experiments

We have studied with interest the experiments in which calcium and phosphorus metabolism have been followed in mature animals not subject to any special demand for these elements, and in mature milking animals   We do not think it worth while to give

any detailed discussion of the first of these classes of experiments. As was to have been expected, both calcium and phosphorus balances are sometimes positive and sometimes negative, but the figures are usually small, and there is no very marked general tendency for either element to be gained or lost faster than the other   It does not seem to us that these results have any bearing, one way or the other, on the view which we have gained from our study of the results on growing animals.   In mature animals which are neither pregnant nor lactating, the assimilative requirements for calcium and phosphorus are small in comparison to those met by the young animal and by the pregnant or lactating female, and it is not to be expected that the mildly unusual conditions which accompany a well conducted balance experiment could show themselves very definitely as a reduction in calcium assimilation [12, 14]   But the results which have been obtained from milking animals seem to us to lend strong support to the view that the conditions of balance experiments interfere with the absorption[15] of calcium and to be incomprehensible on any other basis

We are familiar with experiments by three independent sets of investigators, in which the calcium and phosphorus balances were followed in milking cows [1, 16]   In two of these investigations, namely, those of Anger and Forbes, most of the cows were fed on what might be called normal average rations, and the results can be conveniently considered together.   Both calcium and phosphorus balances were generally negative, and the authors conclude that it is usual for milking cows to be taking these elements from their bodies in order to put them into the milk

It is natural to suppose that the calcium and phosphorus lost by the cows in Anger's and in Forbes' experiments came from the bones.   The calcium must have come chiefly from the bones, for

[14] Experiments of this kind have been carried out by Hofmeister, V , *Landw Ver -Stat* , 1873, xvi, 343   Wellman, O , *Arch ges Physiol* , 1908, cxxi, 508   Sherman, H C , Gillett, L H , and Pope, H M , *J Biol Chem* , 1918, xxxiv, 373   Sherman, H C , Wheeler, L , and Yates, A B , *ibid* , 383

[15] We think it better to use the word "absorption" for milking animals, and to reserve the term ''assimilation'' for the cases where the absorbed material is actually retained in the body

[16] Anger, A , Inaugural dissertation, Bonn, 1898   Hart, E B , McCollum, E V , and Humphrey, G C , *Am J Physiol* , 1909, xxiv, 86

the calcium content of the mammalian soft tissues is so small that the subjects of these experiments often lost in 3 or 4 days more calcium than is contained in the whole body of a cow outside of her bones and teeth

The $\frac{Ca}{P}$ ratio in bone is about 2 15   But the average daily calcium loss divided by the average daily phosphorus loss in Anger's and in Forbes' experiments deviates largely from this ratio.   In Anger's experiments it is 12 23; and in the three sets of experiments reported by Forbes[1] it is 9.07, 213.00, and 3 62 respectively.   The calcium loss is largely in excess of what it should be if the process is simply a wasting of bone tissue.

The results are very difficult to reconcile with our results, which appear to show that pregnant cows readily absorb enough phosphorus from their food to supply the growing embryo, but that they are usually compelled to supply calcium to their offspring from their own bones.   As many cows are always either pregnant or milking, or both, through a large proportion of their lives, these results taken together would seem to point to the impossible conclusion that the normal milch cow is constantly changing the $\frac{Ca}{P}$ ratio of her body in the direction of more phosphorus and less calcium throughout the whole period of her useful life.

The experiment of Hart, McCollum, and Humphrey has been included in this discussion because of the very surprising nature of the conclusions to be drawn from it on the supposition that the calcium and phosphorus metabolism of cows is not disturbed by the collection of their excreta   A single milking cow was kept for 110 consecutive days on known rations low in calcium, and the excreta were collected during 25 days of this period so that the calcium and phosphorus balances could be followed   If it is supposed that the results obtained in the collection periods can be applied to the whole 110 days, it would follow that during this period the cow lost about 25 per cent of all the calcium contained in her body and remained very nearly in phosphorus equilibrium. We should have to suppose, therefore, either that the composition of her bones was profoundly altered, or that 25 per cent of the phosphorus lost from the bones along with the calcium was stored in the soft tissues.

Weiske[17] has studied the bones of a milking goat which was kept for 50 days on a low calcium ration. His results show that the change in the $\frac{Ca}{P}$ ratio of the bones produced by this treatment is negligible   And Hart, McCollum, and Fuller[18] have furnished excellent evidence against the possibility of producing any considerable changes in the phosphorus content of the mammalian soft tissues. It seems to us, therefore, that the experiment of Hart, McCollum, and Humphrey[16] can best be interpreted by supposing that their figures for calcium and phosphorus metabolism during the collection periods are not representative for the whole 110 days of the experiment, and that in the intermediate periods the cow largely made up the marked calcium deficiency produced during the collection periods

The situation may be summed up as follows   There is an apparent contradiction between the results obtained regarding the calcium and phosphorus balances of growing animals and what is known regarding the calcium and phosphorus content of the bodies of the same species of animals.   There is a similar contradiction between results that have been obtained regarding the calcium and phosphorus balances of milking animals, and results obtained from studying the bones of milking animals.   Finally, the results which have been obtained regarding the calcium and phosphorus balances of milking cows appear to be irreconcilable with those which have been obtained regarding those balances in cows toward the end of pregnancy   All these difficulties would be removed by making the assumption that the conditions to which animals have been subjected in balance experiments are likely to interfere with calcium absorption.

We are the more ready to make this assumption, because it is in full agreement with recent striking scientific evidence in regard to the effects of emotional disturbance on the digestive processes   It has been shown by numerous investigators that comparatively mild disagreeable stimuli are capable of producing disturbances in the nervous system which may inhibit the secretion of the

[17] Weiske, H , Z Biol , 1871, vii, 179, 333

[18] Hart, E B , McCollum, E V , and Fuller, J. G., Univ. Wisconsin Agric Exp. Station, Research Bull 1, 1909

digestive juices and the movements of the alimentary tract for considerable periods. A good review of the subject with references to the literature is given by Cannon.[19]

Our Own Evidence in Regard to Disturbance and Calcium Assimilation

Throughout our own experiments, two 4 day collection periods followed each other without any change at all in the experimental routine; and in each case, except that of No. 66, the two collection periods followed a preliminary 10 day period without any change in routine except the introduction of the collection of urine and feces. If the collection of urine and feces produced no disturbance in metabolism, the metabolic behavior of the cows should have been the same in each case in the first of the 4 day collection periods as in the immediately following one; and the same, as far as it was followed, in the preliminary periods as in the collection periods. This was not the case.

It has been our experience that any slight change in the surroundings of a cow or disturbance of her habits is likely to cause a reduction in the amount of water which she will drink. The water taken by Nos 51 and 63 in the collection periods, March 3 to 10, in the intermediate period, March 11 to 20, and in the collection periods, March 21 to 28, was followed (Table II). Both cows took decidedly more water in the intermediate period than in any of the collection periods

Much more evidence pointing in the same direction can be obtained by comparing the metabolic processes of the cows in the first collection periods and in the immediately succeeding ones But, before discussing this evidence, it must be pointed out that certain accidents occurred in the course of the experiments which must be taken into account in interpreting the results

No. 63 developed abrasions during the preliminary period, February 17 to March 2, and refused to lie down during the first 60 hours of the collection period, March 3 to 6. On the evening of March 5, she was provided with a softer bed, and there was no trouble after that Further, No. 51 could not be used in

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[19] Cannon, W B, Bodily changes in pain, hunger, fear and rage, New York and London, 1915, Chapter I, 1–20

the experiment after March 21; No. 66 was unexpectedly substituted for her, and went through the two collection periods, March 21 to 24 and 25 to 28, without any preliminary period in the experimental surroundings. In her case, therefore, the additional factor of new surroundings was added to that of the mere collection of urine and feces.

Table II shows that No. 51 drank more water from March 3 to 6 than from March 7 to 10; and No. 63, more from March 21 to 24 than from March 25 to 28. These results suggest that the collection of urine and feces produced more disturbance during the second of the two consecutive 4 day collection periods than during the first one. It is true that No. 63 drank more water from March 7 to 10 than from March 3 to 6; and No 66, more from March 25 to 28 than from March 21 to 24 But neither of these results is really out of harmony with the suggestion indicated above. In the case of No 63 an additional disturbing factor was introduced during the period March 3 to 6 by the fact that the abrasions which had developed on her hips and shoulders prevented her from lying down. The case of No. 66, on the other hand, may well be discarded from the discussion, for, in addition to the fact that she had had no preliminary period in the experimental stall, the water which she drank on March 21 could not be determined.

The manner in which our cows took their water, therefore, suggests that a disturbance was produced by the collection of their urine and feces, which was cumulative for a considerable period and produced more marked effects in the second 4 days of collection than in the first 4. This suggestion is strongly confirmed by a study of the nitrogen, calcium, and phosphorus balances given in Table X. In every case, except that of No 63, March 3 to 10, where the exceptional disturbance was accidentally introduced in the first 4 days, the assimilation of all three elements occurred more rapidly in the first of the 4 day collection periods than in the immediately succeeding one. In the case of No 63, March 3 to 10, the relations are reversed for all three elements.

It seems surprising perhaps at first sight that assimilation was generally better in the first than in the second 4 day collection period. It might have been expected that the cows would be more accustomed to the collection of their excreta in the second period, and that their assimilative processes would therefore

have been more nearly normal. But we can see no escape from the facts, and the circumstance that the relations are reversed in the case of No. 63, March 3 to 10, seems to us decidedly to strengthen the case. Steenbock and Hart[20] have reported results which show how persistent may be the tendency toward a negative calcium balance under the conditions of a balance experiment, and how this tendency may be overcome by a short period of relief from these conditions.

Our results are in agreement with those of past experiments in showing that the assimilation of calcium is more quickly and more profoundly affected by the experimental procedure than that of either phosphorus or nitrogen It has already been pointed out that the phosphorus assimilation in Nos 51 and 63 was about enough on the average to supply the phosphorus needed by their growing unborn calves, but that the calcium assimilation was never sufficient for this in any single period. It must be pointed out in addition that the fluctuations in calcium assimilation as between the first and second 4 day periods were greater both relatively and absolutely in every case than' the similar fluctuations for either phosphorus or nitrogen.

The Effects on Calcium, Phosphorus, and Nitrogen Assimilation of Feeding Sodium Phosphate and Alternating the Grain and Hay Rations.

For convenience the rations fed to No. 63, March 3 to 10, and to No 66, March 21 to 28, will be called the "experimental rations," while the rations fed to No. 51, March 3 to 10, and to No. 63, March 21 to 28, will be called the "control rations "

The results indicate that phosphorus assimilation is favored by feeding the experimental rations. Although it is clear from certain aspects of the results that phosphorus assimilation is interfered with by disturbance, and that the cows on the experimental rations were decidedly more disturbed than those on the control rations, nevertheless, the cows fed the experimental rations assimilated phosphorus uniformly more rapidly than the controls

In regard to calcium assimilation the results are not so consistent but on the whole they favor the view that this process also occurs

[20] Steenbock, H., and Hart, E B., *J. Biol Chem.*, 1913, xiv, 59

more rapidly in the cows fed the experimental rations   It is true
that the most rapid calcium assimilation occurs in the case of
No. 51, March 3 to 10, on the control ration   But this cow was
decidedly less disturbed by the experimental conditions than any
of the others, and she was, in addition, the largest, the most
robust, and the best milker of the three.   All the other results
are consistent in indicating that calcium assimilation occurs
more rapidly during the experimental feeding   No. 63, for
instance, was assimilating calcium more rapidly during the
experimental period, March 3 to 10, than during the control
period, March 21 to 28, although she was much more disturbed
by the experimental procedure during the first than during the
second of these periods; and even No. 66, though she was not
carrying a calf, and was evidently much upset by the double
disturbance of new surroundings and collection of her urine and
feces during the period March 21 to 28, nevertheless, assimilated
calcium more rapidly on the experimental rations during this
period than did No. 63 during the same period on the control
rations.

The results appear to indicate that the experimental feeding is
unfavorable to nitrogen assimilation.   But we have kept cows
for long periods on the experimental rations, and have found
that they gained weight at least as well and remained in at least
as good general condition as others on the control rations (page
471).   We do not think, therefore, that the experimental rations
are unfavorable to nitrogen assimilation, and we are inclined to
attribute the reduced nitrogen assimilation of No 63 on these
rations to the facts that, when on these rations, she was at an
earlier stage of pregnancy and more disturbed by the experimental
procedure.   That No 66 should have assimilated nitrogen as
fast as the other two cows is not to be expected, as she was not
carrying a calf, and the fact that her nitrogen balance was positive
at all indicates that the experimental feeding is not unfavorable
to nitrogen assimilation

Calcium and Phosphorus in Food, Blood, and Urine.

In Table XII we have brought together our results on urine
and on blood plasma collected approximately simultaneously.
The results there given indicate that there is a connection between

the phosphate feeding, the concentration of inorganic phosphorus in the blood plasma, and the amount of phosphorus excreted in the urine. No. 63, for instance, during the period March 3 to 10, when she was fed phosphate, had an average concentration of about 0 0063 per cent phosphorus in her plasma and was excreting about 0.88 gm. daily in her urine During the period March 21 to 28, when she received no phosphate, her plasma phosphorus dropped to 0 0052 per cent and her daily urinary phosphorus excretion to about one-ninth of what it was previously. No. 51, March 3 to 10, received no phosphate, had a plasma phosphorus of about 0 0056 per cent, and was excreting less than 0 1 gm. of phosphorus in her daily urine.

Closer examination of the figures, however, shows that the urinary excretion of phosphorus does not depend entirely on the concentration of inorganic phosphorus in the plasma No. 66, March 21 to 28, for instance, had a decidedly higher plasma phosphorus than either No 51, March 3 to 10, or No. 63, March 21 to 28, and was, nevertheless, excreting less phosphorus in her daily urine. And if the figures given for No 63 for February 17, March 3, and March 11 are examined, it will be found that there is an inverse relation between the plasma phosphorus and the daily urinary phosphorus excretion

We are inclined to suspect that these disturbances of the relation between the concentrations of plasma phosphorus and urinary phosphorus were connected with differences in the acid-base equilibrium of our animals; but, in the present inadequate state of our knowledge, we do not think it worth while to discuss this matter further.

We obtained wide variations in the amount of calcium excreted in the urine, in spite of the fact that all our cows at all times received about the same amount of calcium in their rations, and that there was little variation in the concentration of calcium in their blood plasma. In looking for an explanation for these variations, we have been struck by the fact that there is an inverse relation between the concentrations of urinary calcium and phosphorus in our experiments, and we think that this circumstance has some significance. Bertram[4] and Rudel[21] have reported

[21] Rudel, G., *Arch. Exp. Path u. Pharmacol.*, 1894, xxxiii, 79.

results which indicate a tendency toward inverse relationship between the calcium and phosphorus of mammalian urine, and it is of course impossible that an alkaline fluid such as average cow's urine could hold much calcium and phosphate in solution at the same time. •

But we do not think it worth while to discuss the mechanism of these relationships at the present time   The chief value of the results which we are considering lies in the demonstration that the amount of calcium excreted in the urine is largely independent of the concentration of calcium in the blood plasma

SUMMARY.

1. The separate collection of urine and feces by attendants, as practiced in balance experiments on cows, produces a nervous disturbance in the animals which interferes markedly with the assimilation of calcium, and, to a less degree, with that of nitrogen and phosphorus. A critical examination of the results of balance experiments carried out in the past indicates that, in the great majority of instances, the experimental procedure has interfered with calcium assimilation.

2. The assimilation of phosphorus by pregnant cows, and probably that of calcium also, is favored by adding disodium phosphate to the grain and feeding the grain and hay of the ration on alternate days.

3 The urinary excretion of phosphorus is markedly influenced by the concentration of inorganic phosphorus in the blood plasma. But our results show that it is influenced also by another factor, which may be connected with the acid-base equilibrium of the body.

4. In our experiments there has been an inverse relation between the amounts of calcium and phosphorus excreted in the urine. But we have no doubt that this relation is easily disturbed by other influences, particularly the relation between the acids and bases of the ration.

TABLE I.

Weights of Cows

Date	No 51.	No 63	Date	No 51.	No. 63
1919	*kg.*	*kg*	*1919*	*kg*	*kg*
Feb. 14	529 5	478 6	Mar 18	540 9	462 3
" 15	515 5	481 4	" 19	542 6	461 4
" 16	514 1	479 1	" 20	550 0	461 4
Average	519 7	479 7		544 5	461 7
Feb 26	524 1	465 5			
" 27	529 1	465 9			
" 28	525 9	467 7			
Average	526 4	466 4		'	
				No 66	
Mar 11	540 5	461 4	Mar 29	397 7	481 8
" 12	542 7	460 0	" 30	408 6	476 8
" 13	538 6	461 4	" 31	401.8	473 6
Average	540 6	460 9	. . .	402 7	477 4

TABLE II.

Water Drunk by Cows '

Date	No 51	No 63.	Date	No 51	No 63.
1919	kg	kg	1919	kg	kg
Mar. 3	20 41	19 50	Mar. 19	26 62	20 41
" 4	16 19	5 10	" 20	21 63	20 01
" 5	20 95	12 22			
" 6	21 60	17 55			
Average	19 79	13 59		23 88 •	21 07
				No 66	
Mar 7	16 92	20 13	Mar 21	Undetermined †	16 87
" 8	0 00	20 07	" 22	0 00	16 36
" 9	18 65	6 38	" 23	13 89	24 58
" 10	13 55	23 95	" 24	7 85	16 84
Average	12 28	17 63		7 25‡	18 66
Mar 11	13 58	17 86	Mar 25	18 82	9 07
" 12	31 35	18 09	" 26	16 78	27 47
" 13	27 07	23 22	" 27	13 15	15 88
" 14	21 94	20 04	" 28	12 02	17 97
" 15	26 11	24 55			
" 16	22 96	21 43			
" 17	26 42	25 46			
" 18	21 12	19 67			
Average				15 19	17 60

* The water supplied to the cows was tap water. Samples of it were analyzed for calcium and phosphorus, and it was found to contain 0 9 mg of calcium per liter, and only a trace of phosphorus The phosphorus content was certainly less than 0 005 mg per liter As none of the cows ever took as much as 30 liters of water in a day, the calcium and phosphorus which they got from the water may be disregarded in calculating the balances.

† No 66 had free access to a water trough during the early morning of March 21, and in the afternoon she drank 10 55 kg of water

‡ Average calculated from water drunk during last 3 days of test

TABLE III

Feed Offered to Cows *

Date	No 51			No 63.		
	Grain without phosphate †	Alfalfa hay	Corn silage	Grain with phosphate ‡	Alfalfa hay	Corn silage
1919	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Feb 17	1,361	1,814	13,608	0	3,628	13,608
" 18	1,361	1,814	13,608	2,722	0	13,608
" 19	1,361	1,814	13,608	0	3,628	13,608
" 20	1,361	1,814	13,608	2,722	0	13,608
,	Same till Mar. 1.			Same till Mar 1		
Mar. 1	1,361	1,814	13,608	0	3,628	5,443
" 2	1,361	1,814	13,608	2,722	0	10,886
" 3	1,361	1,814	13,608	0	3,628	10,886
	Same till Mar. 11			Same till Mar 11.		
Date	Grain with phosphate ‡	Alfalfa hay	Corn silage	Grain without phosphate †	Alfalfa hay	Corn silage
1919	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Mar. 11	0	3,628	13,608	1,361	1,814	10,886
" 12	2,722	0	13,608	1,361	1,814	10,886
	Same till Mar 21			Same till Mar 21		
Date	No 66			No 63		
	Grain with phosphate ‡	Alfalfa hay	Corn silage	Grain without phosphate †	Alfalfa hay	Corn silage
1919	*gm*	*gm.*	*gm*	*. gm*	*gm*	*gm*
Mar 21	0	3,628	13,608	1,361	1,814	10,886
" 22	2,722	0	10,886	1,361	1,814	10,886
" 23	0	3,628	10,886	1,361	1,814	10,886
	Same till Mar 29.			Same till Mar 29		

* From Jan 31 to Feb 16 inclusive No 51 had received the same ration as that given Feb 17 to Mar 11, No 63, from Feb 3 to 16, the same as from Feb 17 to 28, No 66, from Dec. 18, 1917, to Mar 20, 1919, inclusive, the same as from Mar. 21 to 28, except that throughout the earlier period her daily silage was 13,608 All three cows had eaten satisfactorily the rations they received before being put on the experiment

† Grain without phosphate composed of 50 parts of corn and cob meal, 40 parts of wheat bran, 20 parts of cottonseed meal, and 1 part of NaCl

‡ Grain with phosphate composed of 50 parts of corn and cob meal, 40 parts of wheat bran, 20 parts of cottonseed meal, 1 part of NaCl, and 10 parts of Na_2HPO_4 with about 9 molecules of water of crystallization

TABLE IV

Feed Refused by Cows, Totals for Periods *

Period	No 51	No 63
1919		
Feb 17–Mar 2	0	Approximately 900 gm. of grain, 100 gm of hay, 4,000 gm. of silage
Mar 3–6	0	6,237 gm of a mixture of hay and silage
" 7–10	0	Approximately1 ,300 gm of a mixture of hay and silage
" 11–20	0	Negligible quantities; not weighed
	No 66	No 63
Mar 21–24	Approximately 3,000 gm of pieces of cob and stem	Approximately 300 gm of a mixture of hay and silage
" 25–28	Approximately 2,500 gm of pieces of cob and stem	Approximately 1,200 gm of a mixture of hay and silage.

* The feed refused was, in most cases, dried without being weighed There was no object in obtaining its moisture content as it had always lain for some time in the manger, and contained, therefore, a different amount of moisture from the feed originally offered   The dried refused feed was weighed and analyzed for nitrogen, calcium, and phosphorus (Table VI) The figures give a fairly accurate idea of the proportions of grain, hay, and silage contained in the various samples of feed refused

TABLE V.

Composition of Materials Fed

Feed	Water	Nitrogen	Calcium	Phosphorus
	per cent	*per cent*	*per cent*	*per cent*
Grain with phosphate .	17 59	2 1036	0 0659	1 4727
" without "	13 84	2 1837	0 0707	0 7007
Hay . .	16 73	1 8754	1 3103	0 2138
Silage { Mar 3–6	66 91	0 3951	0 1035	0 0733
" 7–10 ..	69 21	0 3400	0 0926	0 0650
" 21–24	69 98	0 3321	0 1001	0 0671
" 25–28	67 45	0 3102	0 1037	0 0686

TABLE VI

Nitrogen, Calcium, and Phosphorus in Feed Refused   Absolute Quantities of Elements in Daily Average Quantities of Feed Refused.

Cow No	Period	Nitrogen	Calcium	Phosphorus
	1919	*gm*	*gm*	*gm*
63	Mar 3–6 .. .	8 99	3 4975	1 5328
	" 7–10 . . .	2 38	1 0963	0 4866
	" 21–24 . .	0 69	0 3566	0 0991
	" 25–28. .	2 75	1 4264	0 3962
66	Mar 21–24 . . .	2 71	0 7559	0 6819
	" 25–28 . .	1 81	0 0218	0 5770

TABLE VII

Absolute Quantities of Feces and Urine Voided during Collection Periods

Date	No 51.		No 63	
	Feces	Urine	Feces	Urine
1919	gm	gm	gm	gm
Mar 3	19,674	4,593	15,422	4,352
" 4	18,031	4,905	17,151	4,678
" 5	16,188	5,103	11,879	4,649
" 6	13,721	4,536	12,786	3,572
Total	67,614	19,137	57,238	17,251
Average	16,903	4,784	14,309	4,313
Mar 7	15,082	4,196	17,436	0
" 8	14,260	4,990	15,196	6,861
" 9	14,571	4,593	17,123	3,062
" 10	14,006	6,832	16,642	3,317
Total	57,919	20,611	66,397	13,240
Average	14,480	5,153	16,599	3,310

Date	No 66		No 63	
	Feces	Urine	Feces	Urine
1919	gm	gm.	gm	gm.
Mar 21	13,749	6,124	13,919	3,515
" 22	10,801	3,147	15,138	3,487
" 23	11,992	4,253	17,549	3,260
" 24	13,580	3,062	17,436	3,175
	50,122	16,586	64,042	13,437
	12,530	4,146	16,010	3,359
Mar. 25	13,636	3,742	20,355	3,600
" 26	11,850	3,544	18,257	3,969
" 27	11,312	4,026	15,649	3,856
" 28	12,219	3,544	18,285	1,673
	49,017	14,856	72,546	13,098
	12,254	3,714	18,136	3,274

TABLE VIII

Composition of Moist Feces

Period	No 51				No 63			
	Water	Nitro-gen	Cal-cium	Phos-phorus	Water	Nitro-gen	Cal-cium	Phos-phorus
1919	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Mar. 3–6	85 86	0 2950	0 2037	0 1178	85 45	0 3212	0 2200	0 1820
" 7–10	85 09	0 3095	0 2438	0 1390	86 63	0 2912	0 1925	0 1560
	No 66				No 63			
Mar 21–24	83 84	0 3441	0 2393	0 1881	86 19	0 2921	0 2017	0 1004
" 25–28	82 66	0 3608	0 3043	0 2436	85 91	0 2839	0 1984	0 1017

TABLE IX

Composition of Urine.

Period	No 51				No 63.			
	Specific gravity	Nitro-gen	Cal-cium	Phos-phorus	Specific gravity	Nitro-gen	Cal-cium.	Phos-phorus
1919		*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	*per cent*
Mar. 3–6 .	1.037	1 0824	0 0183	0 0019	1 038	1 0832	0 0007	0 0208
" 7–10. .	1 035	0 9928	0 0183	0 0016	1 038	1 3196	0 0009	0 0263
	No 66				No 63			
Mar 21–24 .	1 042	1 2311	0 0121	0 0020	1 037	1 0789	0 0016	0 0032
" 25–28	1 045	1 4203	0 0090	0 0021	1 036	0 9352	0 0013	0 0031

TABLE X

Average Daily Intake, Output, and Balance of Nitrogen, Calcium, and Phosphorus.

Cow No	Period	Nitrogen Food Feces Urine Balance	Calcium Food Feces Urine Balance	Phosphorus Food Feces Urine Balance
	1919	*gm*	*gm*	*gm*
51	Mar 3–6	117 50 49 87 51 78 +15 85	38 81 34.43 0.88 +3 50	23 39 19 91 0 09 +3 39
51	Mar 7–10	110 01 44 81 51 16 +14 04	37 33 35 30 0 94 +1 09	22 27 20 13 0 08 +2 06
63	Mar. 3–6	96 66 45 96 46 72 +3 98	32 42 31 48 0 03 +0 91	30 37 26 04 0 90 +3 43
63	Mar 7–10	97 29 48 34 43 68 +5 27	33 63 31 95 0 03 +1 65	30 51 25 89 0 87 +3 75
66	Mar 21–24	98 36 43.12 51 04 +4 20	35 47 29 99 0 50 +4 98	30 99 23 57 0 08 +7 34
66	Mar. 25–28	94 61 44 21 52 75 −2 35	35 32 37 29 0 33 −2 30	30 85 29 85 0 08 +0 92
63	Mar 21–24	99 20 46 77 36 24 +16 19	35 27 32 29 0 05 +2 93	20 61 16.07 0 11 +4 43
63	Mar 25–28	94 76 51.49 30 62 +12 65	34 59 35 98 0 04 −1 43	20 49 18 45 0 10 +1 94

TABLE XI

Composition of Blood Samples

Cow No	Date	Total blood P per 100 gm	Blood corpus- cles	Plasma			
				Phosphorus			Calcium per 100 gm
				Total per 100 gm	Lipoid* per 100 gm calcu- lated	Inor- ganic per 100 gm	
	1919	*mg*	*vol per cent*	*mg*	*mg*	*mg.*	*mg.*
51	Feb 17	15 7	34 6	9 9	4 9	5 0	Not determined.
	Mar. 3	15 9	34 1	10 1	4 3	5 8	" "
	" 11	15 3	32 5	9 6	4 2	5 4	9.5
63	Feb. 17	18 3	38 9	12 3	5 2	7 1	Not determined
	Mar 3	18 2	41 0	11 5	5 3	6 2	" "
	" 11	17 4	40 4	11 9	5 4	6 5	9 7
	" 21	17 2	39 4	9 6	4 4	5 2	9 6
	" 29	17 5	42 6	10 3	5 0	5 3	9 5
66	Mar. 21	18 1	40 4	11 9	6 0	5 9	10 1
	" 29	17 9	45 8	12.1	6 1	6 0	9 7

*Lipoid phosphorus calculated by subtracting inorganic from total (Meigs, E B , Blatherwick, N R , and Cary, C A , *J. Biol Chem* , 1919, xxxvii, 10–17).

TABLE XII

Calcium and Phosphorus in Urine and Blood Plasma *

Cow No	Date	Plasma		Urine			
		Calcium	Inorganic phosphorus	Calcium	Phosphorus	Daily calcium excretion	Daily phosphorus excretion
	1919	per cent	per cent	per cent	per cent	gm	gm
	Feb. 17	Not determined	0 0050	Not determined	0 0017	Not determined	0 0689
51	Mar 3	" "	0 0058	0 0183	0 0019	0 8755	0 0907
	" 11	0 0095	0 0054	0 0183	0 0016	0 9425	0 0818
	Feb. 17	Not determined	0.0071	Not determined.	0 0099	Not determined	0 3817
	Mar 3	" "	0 0062	0 0007	0 0208	0 0303	0 8979
63	" 11	0 0097	0 0065	0 0009	0 0263	0 0293	0 8706
	" 21	0 0096	0 0052	0 0016	0 0032	0 0543	0 1093
	" 29	0 0095	0 0053	0 0013	0 0031	0 0423	0 1006
66	Mar. 21	0 0101	0 0059	0 0121	0 0020	0 5000	0 0827
	" 29	0 0097	0 0060	0 0090	0 0021	0 3340	0 0774

*In this table the concentrations of calcium and phosphorus in the blood plasma are taken as corresponding to the concentrations in the urine of the nearest collection period.   For instance, the plasma obtained Mar 3 is taken as corresponding to the urine collected in the period Mar 3–6; the plasma obtained Mar 11, to the urine collected Mar. 7–10, and so on On Feb 17, 18 hour samples of urine were specially collected to compare with the blood samples obtained on that date

FAT-SOLUBLE VITAMINE.*

II. THE FAT-SOLUBLE VITAMINE CONTENT OF ROOTS, TOGETHER WITH SOME OBSERVATIONS ON THEIR WATER-SOLUBLE VITAMINE CONTENT.

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WITH THE COOPERATION OF M. T SELL

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PLATE 1

In the evolution of the modern conception of the dietary needs of the body there has come an appreciation of the significance of small amounts of those indispensable nutritive factors known as vitamines, but unfortunately with this appreciation there has been aroused much apprehension in the minds of many in regard to the wisdom of omitting certain foods from the human diet for fear of unknowingly reducing the vitamine content of the diet to dangerous limits   While it is not intended to depre- cate the value of discretion in the selection of the ingredients of the diet for reasons other than satisfying the requirements of palatability, good proteins, and mineral elements, it is deemed rather unfortunate that conclusions should have been hastily drawn and promiscuously applied after only a limited survey of the dietary properties of our naturally occurring foods had been made, and especially when such were made with animals about which we have no information as to their requirements when compared with those of man.   Taking into consideration the apparent limitations imposed on methods of experimental inquiry now in use, what seems most desirable above all else—after the development of sufficient theory to serve as a suitable working basis—is the accumulation of sufficient data which may give an insight into the specific comparative nutritive properties of dif-

ferent foods   Such information seems especially called for by the great interest shown by dietitians in the occurrence and stability of the vitamines.

It is now generally recognized that of the substances which are collectively known as vitamines there are three distinct kinds; namely, the water-soluble or antineuritic vitamine, the fat-soluble or antixerophthalmic vitamine, and the antiscorbutic vitamine.   In the present series of papers it is planned to confine the discussion to the fat-soluble vitamine, but incidentally certain points of interest in connection with the water-soluble vitamine will be mentioned, especially with reference to the determination of the amount present so that it could not possibly be a limiting factor in the performance of the animals on any of the rations which it was desired to test for the relative fat-soluble vitamine content.   Various phases of the water-soluble vitamine question will be published in another connection.

· The specific growth-promoting property which is now associated with the occurrence of the fat-soluble vitamine was first dissected from different factors, though functioning in a similar capacity, when McCollum and Davis[1] in 1913, in a study of the value of different salt mixtures, observed that in a synthetic ration an ether extract of butter or egg yolk had a stimulating action on growth which was not possessed by other fats such as lard or olive oil.   Very shortly thereafter Osborne and Mendel[2] published experiments, some of them of 180 days duration, where it was pointed out that their milk rations had special dietary properties not found in their milk-free rations and that this characteristic seemed to be true of rations carrying an equivalent amount of butter as well   Later[3] they demonstrated in very conclusive experiments, more so than any published up to that time. that this property resided in the butter fat   Furthermore, they substantiated McCollum and Davis' observation that egg yolk fat was efficient in this respect and added that "some other oils" were no more efficient than lard.   In 1914 Osborne and Mendel[4] reported

[1] McCollum, E V , and Davis, M , *J. Biol Chem* , 1913, xv, 167

[2] Osborne, T B., and Mendel, L B , *J Biol Chem* , 1913, xv, 311.

[3] Osborne, T B , and Mendel, L. B , *J Biol Chem* , 1913–14, xvi, 423.

[4] Osborne, T. B , and Mendel, L B , *J Biol Chem* , 1914, xvii, 402

"We have likewise obtained uniform success by substituting cod liver oil for a portion of the lard in our standard diets   In contrast with this are the uniformly observed failures of almond oil to restore growth when it had ceased on the usual lard food mixtures "

It is not intended to give here a résumé of the findings of others with respect to the occurrence of the fat-soluble vitamine in fats, grains, leaves, and other food materials, as that has already been done in a most excellent manner by Osborne and Mendel[5] and by Emmett and Luros,[6] nor do we intend to comment extensively as to the merits of the recorded observations.   It is, however, safe to venture that the time is not yet ripe to warrant general classification of foods into groups rich, poor, and free from fat-soluble vitamine as general methods of experimentation, especially in reference to vitamine content of the basal food ingredients, period of observation, and control of experimental animals, differ so greatly in different laboratories that the conclusions of the different observers are hardly comparable. Furthermore, there is much reason to believe, as will be brought out in later papers, that the variation in the fat-soluble vitamine content of naturally occurring food materials, even when harvested at the same stage of development, is tremendous.   No doubt there lies here the foundation for many interesting correlations in the functional rôle of specific substances in both plant and animal kingdoms, the significance of which can scarcely be predicted.

One correlation that has been advanced is an apparent attempt to associate a richness in vitamine content with the occurrence of a preponderance of actively functioning cells [7]   This deduction would seem to be based on a recognition of the great indispensibility of the vitamines for growth and an assumption that the vitamines are equally indispensable in individual physiological processes, none the less active, but of a highly specialized character.   Suffice it to say that as nothing of the specific internal rôle of vitamines is known outside of the pharmacological findings of Uhlmann,[8] who, however, worked with extracts

[5] Osborne, T B , and Mendel, L B , *J Biol Chem* , 1917, xxxii, 309

[6] Emmet, A D , and Luros, G O , *J Biol Chem* , 1919, xxxviii, 441

[7] McCollum, E V , *J Home Econ* , 1918, x, 195

[8] Uhlmann, F , *Z. Biol* , 1917–18, lxviii, 419, 457

of very complex character, such generalizations appear gratuitous. In this connection a high vitamine content of liver tissue, instead of being due to or associated with its activity as a glandular organ, might with equal propriety be assumed on the basis of its well known functions as a storage organ, or might possibly be attributed to the absorption of substances not indispensable to the organ but absorbed due to an inefficiency of the mechanism which excludes the entrance of substances in amounts greater than the needs as these substances are brought to it in the portal circulation. Similarly, the high vitamine content of the kidney may be said to be due to the temporary retention of the vitamines as unutilizable amounts of them found in the ration are eliminated.

One thing appears reasonably certain· In the plant kingdom the occurrence of the vitamine cannot always be associated with a preponderance of actively functioning cells as distinguished from those that serve primarily as storage organs. This is brought out in an analysis of our data on the occurrence of the fat-soluble vitamine in tubers and roots even though all must be considered storage organs for those constituents that are needed by the growing plant in the following season.[9] The sweet potato, as an example of the tuber, is rich in this vitamine, while the rutabaga and the potato contain relatively little of this constituent. Similarly, among the roots carrots contain much of it as has been suggested by the work of Denton and Kohman,[10] while mangels and sugar beets are poor in it. Results will be discussed in further detail under the various following experimental headings.

EXPERIMENTAL.

The relative amounts of vitamine present in the tubers and roots were determined by incorporating various percentages of them in a basal rat ration which already satisfied all other nutritive requirements, and which was as free from the vitamines as ordinary methods of laboratory manipulation could produce them. We have repeatedly demonstrated that the basal constituents of our rations were too poor in vitamines to influence in any way

[9] Steenbock, H , *Science*, 1918, xlvii, 119
[10] Denton, M C , and Kohman, E , *J Biol. Chem.*, 1918, xxxvi, 249

the conclusions at which we have arrived, but on the other hand we make no claim that very small amounts of some vitamines may still not have been present and thus may have influenced in degree—though not in character—failure in maintenance, growth, reproduction, and rearing of the young as indicated by the experimental rats   This will readily be appreciated on inspection of the charts in the various groups.   When failure in growth or maintenance resulted and this was suspected as being due to a vitamine deficiency this was often incontrovertibly established by adding additional water or fat-soluble vitamine and noting the result.   The water-soluble vitamine was added in the form of an alcoholic extract of ether-extracted wheat embryo evaporated on dextrin, while the fat-soluble vitamine was added as found in butter fat obtained by melting and filtering butter fat at a low temperature.   The dextrin used was partially dextrinized corn-starch prepared by heating the starch with 0.1 per cent citric acid solution in an autoclave for a number of hours and then drying and grinding it.   The casein was prepared from commercial casein by washing it repeatedly with distilled water acidified with acetic acid for an entire week and then drying and pulverizing it.   Salts were incorporated in the rations in the form of artificial salt mixtures prepared from purified reagents.[11]

[11] The salt mixtures used had the following composition,—

Salt 35
NaCl	. 1 0
$CaCO_3$	. 1 5

Salt 1
NaCl	. 0 173
$MgSO_4$ (anhydrous)	0 266
$NaH_2PO_4H_2O$	0 347
K_2HPO_4	0 954
$CaH_4(PO_4)_2H_2O$	0 540
Fe citrate	0 118
Ca lactate	. 1 300

Salt 32·
NaCl	0 202
$MgSO_4$ (anhydrous)	0 311
$Na_2HPO_412H_2O$	0 526
K_2HPO_4	. 1 115
$Ca_2H_2(PO_4)_2H_2O$	1 116
Ca lactate	0 289
Fe citrate	0 138

When these experiments were initiated no data on the heat stability of the fat-soluble vitamine as found in plant materials were available. From the work of McCollum and Davis who showed that this vitamine was still contained in the yolk of hard boiled eggs[12] and from the work of Osborne and Mendel[13] on the stability of the vitamine in butter fat the idea was generally prevalent that this vitamine was thermostable. We[14] did not find this conclusion generally acceptable after we had demonstrated to our satisfaction that though the reaction is one of slow velocity the fat-soluble vitamine in butter fat is destroyed by heat It might be mentioned that this observation has since been substantiated by Drummond [15] As in the materials that we used, the vitamines are in contact with many different compounds and as no data on the stability of the vitamine under these conditions were available we were forced to adopt the procedure of carrying out all our drying operations at a low temperature wherever possible as the roots had to be dried for comminution and introduction into the ration. Usually the roots were dried at room temperature in an air current and later over anhydrous calcium chloride. In isolated instances, as later noted, it was necessary to use higher temperatures and in some instances it was even found necessary to cook the roots to make them digestible. Later on as our experiments progressed we found that the fat-soluble vitamine as found in plant materials was very stable to heat[16] so that we were able to allow ourselves more leeway in the variety of treatments to which the materials were subjected. Variations from the regular procedure are noted and discussed under the various experimental groups.

Fat-Soluble Vitamine in Carrots

The roots were washed free from all extraneous material and then pulped on a power beet rasp. In this condition they dried readily in an air current at room temperature. To secure fine

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[12] McCollum, E V , and Davis, M , *Proc Soc Exp. Biol and Med ,* 1913-14, xi, 101

[13] Osborne, T B , and Mendel, L B , *J. Biol Chem ,* 1915, xx, 381

[14] Steenbock, H , Boutwell, P W , and Kent, H E , *J Biol Chem ,* 1918, xxxv, 517

[15] Drummond, J C., *Biochem J ,* 1919, xiii, 81.

[16] Unpublished data

comminution they were often dried over calcium chloride for a few days after which they could readily be ground to an impalpable powder in a ball mill. Usually, however, grinding in an Excelsior mill was sufficient to pulverize the pulp to a degree which prevented any picking out of the ration by the experimental animals. With the high sugar content of carrots good consumption of a high percentage carrot ration was generally secured. They were fed at levels of 5, 10, 15, 25, and 60 per cent with and without water-soluble and fat-soluble vitamine additions.

Chart 1, Lot 163'-
5% of dried carrots does not furnish enough water-soluble vitamine, nor enough fat-soluble vitamine for growth.

Ration:- Carrots 5, Casein 18, Salts 1 4, Agar 2, Dextrin 71
Period B:- 20 grams of dextrin carrying the alcoholic extract of 80 grams of wheat embryo replaced part of the dextrin.
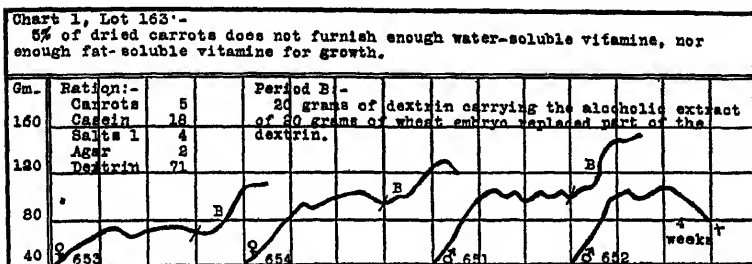
CHART 1 As seen in the chart when a ration satisfactory in all other respects is dependent for its vitamine content on 5 per cent of dried carrots there results partial failure in the growth performance which is not entirely corrected by an addition of water-soluble vitamine in the form of an alcoholic extract of ether-extracted wheat embryo This indicates that this amount of dried carrots does not furnish a sufficiency of either the water or the fat-soluble vitamine Rat 652 had an attack of xerophthalmia after having been on the ration 6 weeks It was, however, but a mild attack as later during its 12th week its eyes were in good condition. At this time and for 3 weeks previously it was losing weight rapidly which appeared to be caused by the insufficiency of water-soluble vitamine as it succumbed to an acute attack of polyneuritis Rat 653 was blinded by xerophthalmia at the end of the 10th week, but no irreparable damage was done by the acute inflammation and 2 weeks later its eyes were entirely normal. We have in this lot an instance where presence of a subnormal amount of one vitamine was apparently brought to light by a similar situation with respect to another vitamine When the deficiency of the water-soluble vitamine was corrected the amount of fat-soluble vitamine originally present was able to allow some further growth to result. This statement can be made on the basis of results brought out later (Charts 8 to 34) which indicate that the amount of fat-soluble vitamine introduced with the water-soluble vitamine could not have been responsible for the temporary response when this was added. It brings out one of the innumerable instances where a tendency to one nutritive deficiency heightens the susceptibility and results in the onset of symptoms resulting from similar or other unfavorable environmental conditions

Chart 2, Lot 164:-
5% of carrots supplemented with fat-soluble vitamine in butter fat was no better for growth than without it; water-soluble vitamine was the primary deficiency.
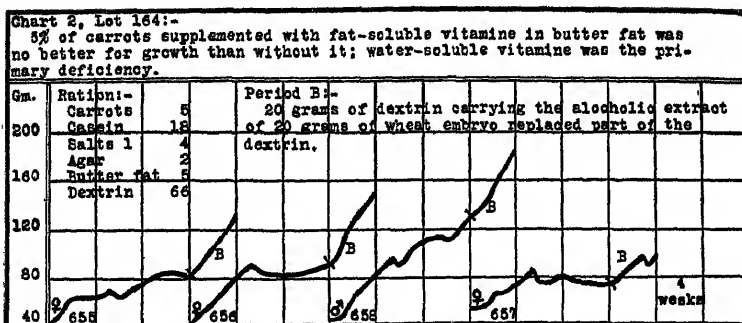
CHART 2   In this lot when the ration was already fortified with additional fat-soluble vitamine in the form of butter fat, while the growth performance was not any better than without this addition, the augmented rapidity of growth was remarkable when additional water-soluble vitamine was added as indicated in Chart 1.   This shows—what had been surmised —that the primary vitamine deficiency in a ration carrying only 5 per cent of dried carrots is due to an insufficiency of the water-soluble vitamine

Chart 3, Lot 162.-
10% of carrots apparently furnished enough water-soluble vitamine as there was no response in growth on adding more.
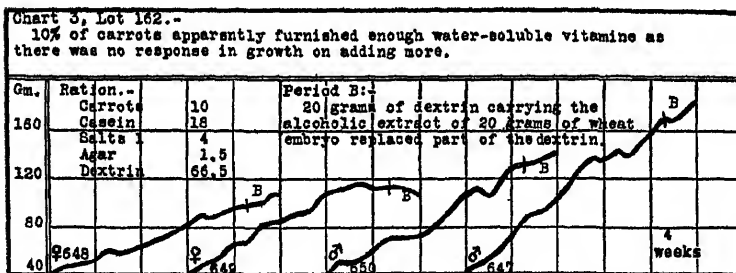
CHART 3   It appears that a ration carrying 10 per cent of dried carrots contains enough water-soluble vitamine to allow considerable growth. This is inferred from the fact that in this lot no response in augmented rapidity of growth resulted when more water-soluble vitamine was added when it was established that even in a ration carrying 5 per cent of carrots the primary deficiency was one of this vitamine   Growth here too was far from normal, but as the animals remained in fine condition during the 20 weeks of experimentation it may be taken as another instance where a ration may not be glaringly deficient in any one dietary factor, but still a suboptimal content of a number of dietetically indispensable constituents may still prevent growth from proceeding at the normal rate.

Chart 4, Lot 109 -
15% of carrots furnishes approximately a sufficiency of both the fat-and water-soluble vitamines for growth.
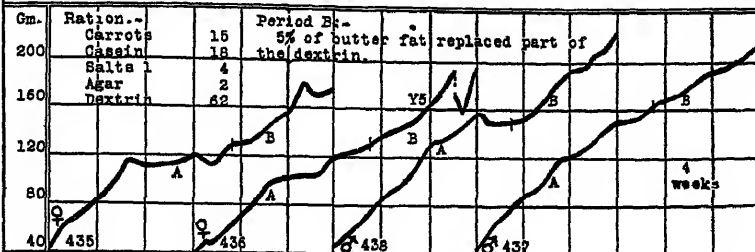
CHART 4 On a ration containing 15 per cent of carrots as the sole source of vitamines, rats are able to continue their growth to maturity at a rate slightly below that possible on our basal ration when suitably supplemented. Rat 436, after the ration had been supplemented with an additional amount of the fat-soluble vitamine, which had no apparent effect on growth, produced a litter of five young While normal in weight—they weighed 25 gm.—they failed to be nourished by the mother rat and soon succumbed An individual failure of this kind has, however, no special significance as regards reproduction.

Chart 5, Lot 268:-
When 15% of carrots was supplemented with additional water-soluble vitamine growth generally was not improved and young were successfully reared.
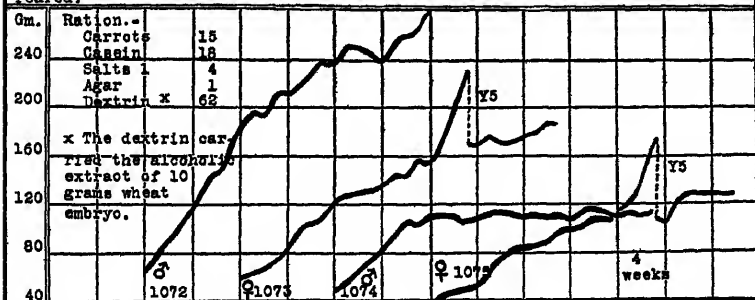
CHART 5. 15 per cent of carrots when supplemented with an additional amount of the water-soluble vitamine did not, with one exception, induce any better growth than when not so supplemented (Chart 3) Rats 1074 and 1075 were decidedly stunted They' gave the impression of being shorter in body length than their weight would normally call for. We have never seen this effect occur in rats where normal growth was disturbed by a lack of vitamines in the diet, but it is a common occurrence where stunting has resulted from digestive disturbances. That the poor growth performance of these animals was not due to a vitamine deficiency is distinctly indicated by the fact that Rats 1073 and 1075 reared five out

of the ten young that they pioduced   In our experience, the quantity of
vitamines required by the rat for normal milk production is considerably
greater than that necessary for normal growth   The young 1eared were,
however, far from being normal as they did not attain an average weight
of 45 gm. until they were 55 days old; normally in our stock this weight
is attained in less than half that time   When weaned, some of the young
were very much bloated which gave us our first evidence that the analysis,
attributing the cause of the small size of Rats 1074 and 1075 to digestive
disturbances rather than to a vitamine deficiency, was probably correct.

Chart 6, Lot 157:-
When 15% of carrots was supplemented with fat-soluble vitamine growth was
somewhat improved (palatibility?) and young were successfully reared.
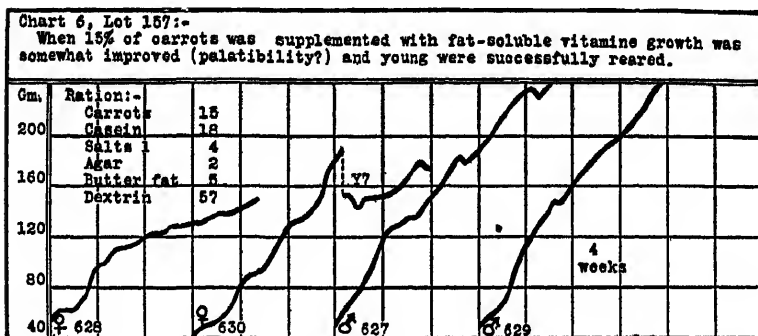
CHART 6. Lot 157 offers additional evidence to that already brought
out in Lot 268, Chart 5, that 15 per cent of carrots introduces enough
water-soluble vitamine into the diet for normal growth requirements and
even for the rearing of some young.   In fact there must have been a con-
siderable excess for growth otherwise young could not have been reared
as conditions in the tract could not be considered normal   To a certain
extent, growth in this lot is better than in Lot 268 where butter fat was
not added and water-soluble vitamine was added instead, but it is not
pronounced enough to be given special significance.

As in Lot 268 young were reared although only two out of the seven and
that at approximately the same subnormal rate, the two together weigh-
ing only 111 gm. when 56 days old.   In the light of this it is certain that the
retarded development of the young was not caused by an insufficiency of
the fat-soluble vitamine.   Furthermore, in none of the animals was
there ever any indication of xerophthalmia.

Chart 7, Lot 219:-
  25% of carrots induced variable growth responses due to resultant
digestive disturbances in some animals.

Gm. Ration:-
       Carrots   25
160   Casein   17
       Salts 1   4
       Agar   1
120   Dextrin   53

 80

 40

♀878   ♂ 876   ♂ 877   ♀ 879   4 weeks
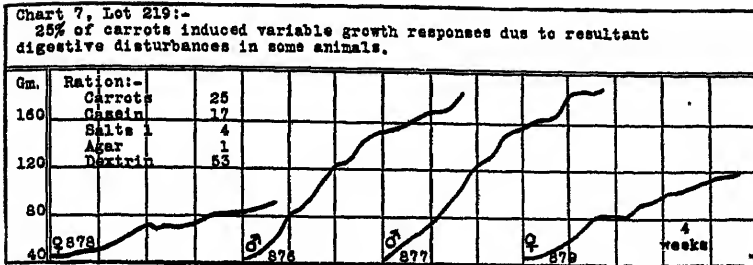
CHART 7. As already indicated by the results pictured in Charts 5 and 6 a high content of carrots in the ration is liable to cause digestive disturbances and as a result of difference in the power of resistance to these disturbances there is considerable variation in the growth performance of different animals. When the amount of carrots is increased to 25 per cent these differences become still more marked as the disturbances are aggravated so that tympanites was regularly observed.

It is to be noted that the results obtained on these carrot rations even when carrying but a low percentage of carrots were never entirely satisfactory from the standpoint of normal nutrition. When the carrot content was too low, the vitamine deficiency would become evident, and if too high or even just high enough for the proper vitamine relations, the large amount of fermentable but indigestible carbohydrate introduced into the ration would cause digestive disturbances and the resultant marked interference with normal growth For this reason it was not considered advisable to introduce more than 25 per cent of carrots into the ration. In one case where as much as 60 per cent of carrots was fed diarrhea and tympanites always resulted and rendered the results difficult of interpretation.

By adopting such a low level of carrots in the diet that the disturbing effects were minimized, we were able to demonstrate that carrots are remarkably rich in the fat-soluble vitamine In spite of growth not being entirely satisfactory, this is the only conclusion that can be arrived at in view of our findings that on as low a level as 15 per cent of carrots as the sole source of the fat-soluble vitamine female rats are able to raise their young without any indications of a deficiency.

Chart 8, Lot 615:-
  The water-soluble vitamine preparations from wheat embryo do not carry
any appreciable amount of the fat-soluble vitamine.

Gm. Ration.-
       Casein   16   x The dextrin carried the alcoholic extract
120   Salts 32   4   of 78 grams of ether-extracted wheat embryo.
       Agar   2
       Dextrin x   78
 80

 40

♂ 2459   ♀ 2456   ♂ 2457   ♀ 2458   4 weeks
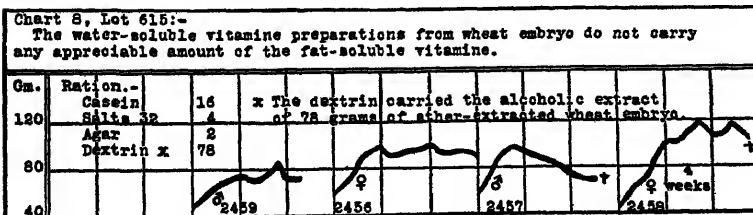
CHART 8. In experiments where additional water-soluble vitamine was added the amount of fat-soluble vitamine added with it was extremely

small as brought out by the growth performance of Rats 2456 and 2459 wheie they received almost three times as much of the preparation as any of the other experimental groups. In this group Rat 2458 died from xerophthalmia and Rats 2456 and 2459 both had inflamed eyes at the time of their death It is not necessary therefore to make any reservations in the conclusions so far drawn with respect to the amounts of fat-soluble vitamine present in those rations where water-soluble vitamine had also been added.

From our data and from what is known of their antiscorbutic properties it appears that the practice of feeding carrots in lieu of green materials in vogue by many small animal breeders is dietetically justified from the vitamine standpoint alone. It is also possible that herein lies experimental justification for the use of carrot juice as an adjuvant to the boiled milk diet of children.

Fat-Soluble Vitamine in Swedes or Rutabagas.

The roots used in these experiments were of a pale yellow color and of excellent quality They were washed, pulped, and then dried in an air current at room temperature and subsequently over calcium chloride to facilitate their comminution in our milling apparatus When fed, exposure to the air and light had bleached them until they were almost void of all yellow color.

Chart 9, Lot 181'—
15% of rutabagas carries plenty of the water-soluble vitamine not only for growth but even for the rearing of young.

Gm.	Ration.—	
	Rutabagas	15
240	Casein	18
	Salts 1	4
	Agar	2
200	Butter fat	5
	Dextrin	57
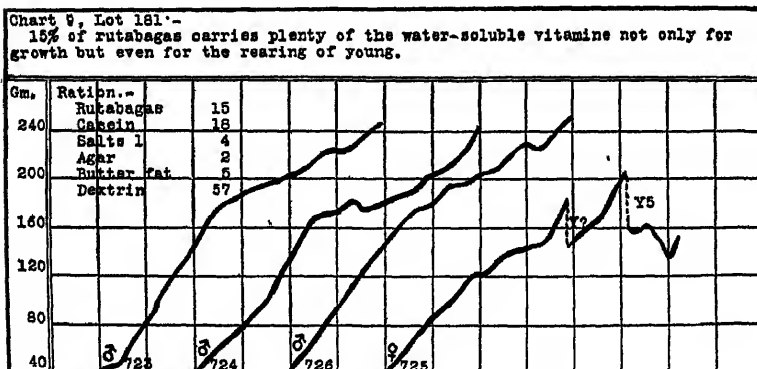
CHART 9. As it is always necessary to insuie a sufficiency of all the dietary requirements outside of the unknown which it is desired to determine, a diet containing 15 per cent of rutabagas suitably supplemented with casein and salts was also supplemented with a sufficiency of the fat-soluble vitamine as found in butter fat to allow determination of the

amount of water-soluble vitamine present. As is seen in the chart, growth was practically normal, furthermore Rat 725 reared her second litter of young to weaning Their growth was not entirely normal, yet they averaged 42 gm. in weight when 33 days old which speaks well for the nutritive sufficiency of the ration. All the rats were in good condition at the time of termination of the experiment, 24 weeks after its inauguration.

Chart 10, Lot 184:-
15% of rutabagas does not carry sufficient fat-soluble vitamine for growth.
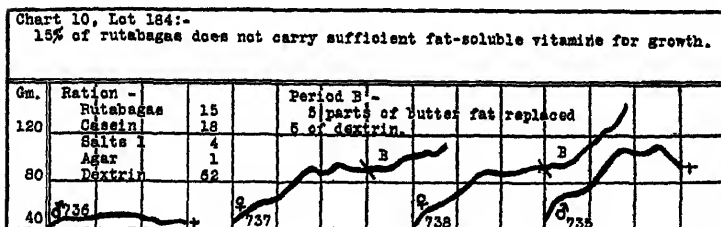
CHART 10 With a sufficiency of the water-soluble vitamine in 15 per cent of rutabagas established (Chart 9), it was comparatively an easy matter to determine its relative fat-soluble vitamine content Unlike carrots (Chart 4) 15 per cent of rutabagas as the sole source of the fat-soluble vitamine leads to early indications of its deficiency Rat 735 became afflicted with xerophthalmia which ultimately, after 12 weeks, was the immediate cause of its death Rat 737 likewise contracted xerophthalmia, but on the addition of fat-soluble vitamine in the form of butter fat, the inflammation disappeared rapidly so that no further signs of it were noted during the last 6 weeks of the experiment; simultaneously it more than maintained its weight and appeared to be in fair nutritive condition. Rat 738, while it had not contracted xerophthalmia, improved remarkably in appearance after the addition of butter fat to its ration. It was short, however, and gave the impression of having been stunted not by a nutritive deficiency, but by digestive disturbances.

Chart 11, Lot 222.-
25% of rutabagas does not carry sufficient amounts of the fat-soluble vitamine for normal growth.
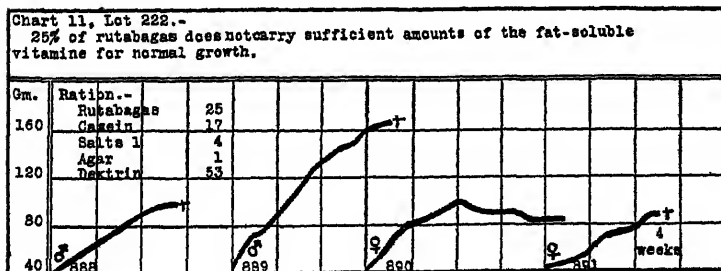
CHART 11 When the amount of rutabagas was increased to 25 per cent of the ration the demands for fat-soluble vitamine still remained unsatisfied This cannot be inferred from the observed failure of normal

growth to result because digestive disturbances attributed to the hemi-cellulose variety of carbohydrates complicated the situation just as in the case of the carrots.   Rat 890 was the only rat that contracted xeroph-thalmia   Rat 889 was bloated almost continually and ultimately died from this condition.   We are inclined to believe that the more pronounced tympanites was due to the greater amount of food consumed by this animal.   If it consumed more food, this would easily explain its more pronounced growth as thereby its vitamine intake was satisfied.   In such instances as this individual records of food consumption would facilitate the interpretation of data.   In most instances, however, accurate con-clusions can be arrived at by the law of averages.   In the case of Rats 888 and 891 no tympanites was observed and at the time of their death their eyes were perfectly normal

Chart 12, Lot 183.—
60% of rutabagas if it carries sufficient fat-soluble vitamine for growth causes too pronounced digestive disturbances to give conclusive indications thereof.
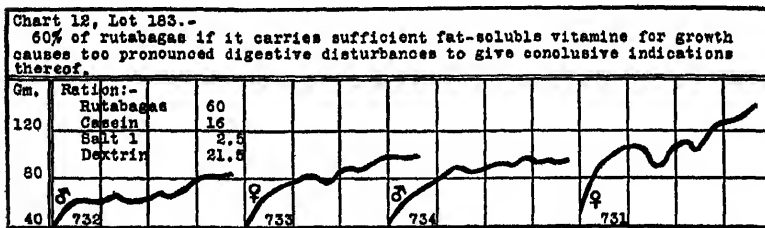
CHART 12   Whether sufficient amounts of fat-soluble vitamine to satisfy the requirements for growth in the rat can ·be introduced into the ration with dried rutabagas is difficult of determination   Whenever large amounts of the root are incorporated in the diet digestive disturb-ance which appeared even when only 25 per cent of the root was fed became so aggravated that normal growth was impossible.   At a level of 60 per cent it is noteworthy that while the diet was rendered distinctly unfavor-able for growth on account of the persistent tympanites and occasional diarrhea no xerophthalmia was observed in any of the animals during the 15 weeks of the experimental period.   If the rutabagas contained none of the fat-soluble vitamine it was to be expected that xerophthalmia would have resulted in the presence of these digestive disturbances and over such a protracted period.   But final conclusions cannot be arrived at with these limited data   There is no question, however, that if ruta-bagas contain any demonstrable amounts of the fat-soluble vitamine they are not to be considered comparable to those found in carrots

Fat-Soluble Vitamine In Dasheens.[17]

The dasheens, both corms and roots of medium size and free from foreign material, were sliced without peeling and then dried

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[17] The common name of dasheen is taro, and the scientific name *Cala-dium colocasia.*

at room temperature in an air current.   In this condition they could be readily ground to a fine powder and incorporated in the ration.   No difficulty in securing consumption was observed.

Chart 13, Lot 581:-
15% of raw dasheens furnishes enough of the water-soluble vitamine for long continued growth.

Gm.	Ration:-	
	Dasheens	15
240	Casein	18
	Salts 1	4
200	Agar	1
	Butter fat	8
	Dextrin	54

160

120

80

40

♂ 2326   ♂ 2324   ♀ 2327   ♀ 2325

4 weeks
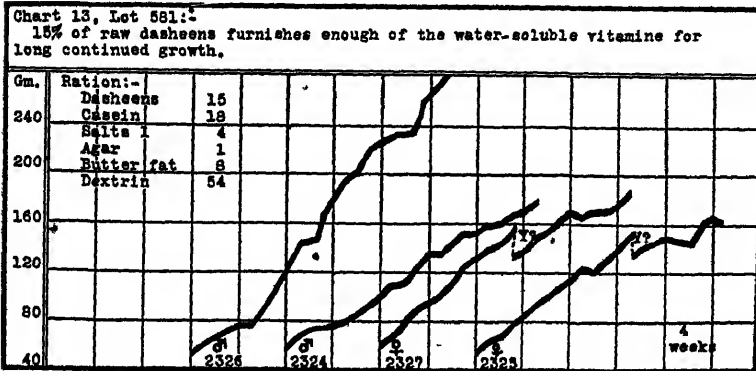
CHART 13   From the performance of the animals in this lot there remains no question but what dasheens are abundantly supplied with the water-soluble vitamine to support long continued growth of the rat.   It is true that the observed growth was not what could be considered normal, but nevertheless for our purposes of comparison the results must be considered very satisfactory especially in view of the fact that no rations which can be considered synthetic in any sense of the word are entirely satisfactory even for growth and certainly not for reproduction and rearing of the young.

Chart 14, Lot 569:-
15% of raw dasheens do not furnish any demonstrable amounts of the fat-soluble vitamine.

Gm.	Ration:-			x The dextrin carried the alcoholic extract of		
120	Dasheens	15		10 grams ether-extracted wheat embryo.		4 weeks
	Casein	18				
	Salts 1	4				
80	Agar	1				
	Dextrin x	62				

40

♀ 2236   ♂ 2237   ♀ 2238   ♂ 2239
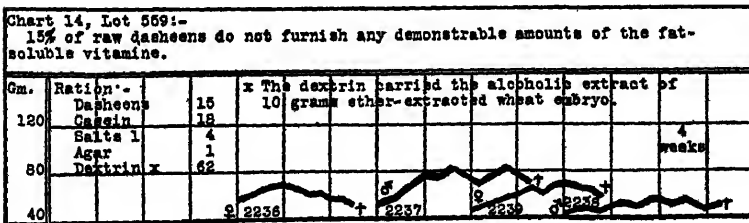
CHART 14.   In a ration provided with a liberal excess of the water-soluble vitamine and all other nutritive factors, outside of the fat-soluble vitamine necessary for growth, provided for, 15 per cent of dasheens does not take care of this deficiency to allow even the minimum amount of growth.   Rat 2339 became blinded by the resultant xerophthalmia but the other rats in their miserable condition became infested with lice, making it difficult to ascertain definitely before their death—which followed shortly—whether or not the inflamed condition of the eyes was due to the fat-soluble vitamine deficiency or to the irritation.   Under the conditions,

the consistent failure of growth together with the occurrence of one clean-cut case of xerophthalmia speaks decisively for a fat-soluble vitamine deficiency in this ration

In several instances attempts were made to feed a higher percentage of dasheens. This resulted in complete failure, but for reasons which had hitherto not been appreciated. When raw dasheens are fed in liberal amounts it becomes evident that they are very difficultly digestible which fact became especially evident when the amount was increased to as high a content as 80 per cent of the rations. Even though the rations were complete in every way the indigestibility of raw dasheen starch prevented the rats from even maintaining themselves. Large quantities of the feed were devoured at times and bulky fecal residues were eliminated due to the excretion of undigested starch as indicated by the iodine potassium iodide reaction. Autopsy of a number of animals indicated that the abdominal distention which had at first been taken as due to gaseous fermentation was in reality due to an abnormal enlargement of the cecum and large intestine in accommodation to the accumulation of the undigested food.

Chart 15, Lot 688.— Even when the dasheens were rendered digestible by cooking, 83.5% in the ration gave no evidence of a sufficient fat-soluble vitamine content.
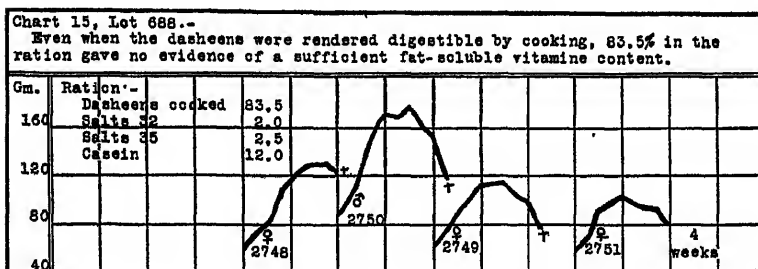
CHART 15 As the indigestibility of the starch precluded the possibility of demonstrating the low fat-soluble vitamine content of dasheens it became necessary to cook them When cooked, difficulty in the digestibility of the starch was no longer observed, but neither was there made evident the presence of a fat-soluble vitamine content Viewed in the light of results obtained later we are thoroughly convinced that [the data are representative of what would have been obtained on the raw dasheens if results had not been obscured by their indigestibility It is probable that in spite of their indigestibility when 15 per cent of the raw dasheens was fed if they had contained any demonstrable amount of the vitamine it would still have been shown by the growth responses as the demonstration of the presence of the water-soluble vitamine was not

interfered with by this condition (Chart 13)   Furthermore the dasheens fed were cooked at a low temperature (5 pounds steam pressure for 20 minutes) and were then dried at room temperature in an air current. From what we now know of the stability of the fat-soluble vitamine in plant materials the amount destroyed must have been very small if at all demonstrable by feeding experiments.   If the fat-soluble vitamine occurs at all in the dasheens it should here have become evident.

Fat-Soluble Vitamine in Red Beets

The red beets of medium size were scrubbed thoroughly, then sliced, and dried in an air current at room temperature.   Under these conditions and even when kept over anhydrous calcium chloride for some weeks they did not dry enough to make it possible to grind them.   Only by drying them in a thin layer for 20 minutes at 90° did they become brittle enough to grind and later pulverize to an impalpable powder in a ball mill.

Chart 16, Lot 586:-
15% of red beets does not furnish demonstrable amounts of the fat-soluble vitamine.
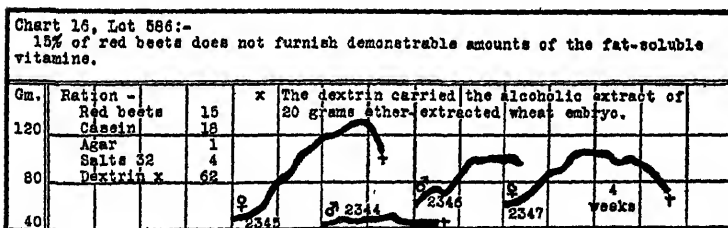
CHART 16   In a ration complete in its content of the dietary essentials requisite for fairly good growth with the exception of the fat-soluble vitamine 15 per cent of red beets does not furnish enough of this vitamine. None of the rats grew at the normal rate for even a limited time and one, Rat 2344, remained absolutely stationary in body weight.   Rats 2345 and 2347 developed bad cases of xerophthalmia which persisted to the time of their death.   Rat 2344 died without any specific symptoms of vitamine deficiency

Chart 17, Lot 699 -
In a complete ration, the introduction of 15% of red beets does not suppress the growth of the animals.
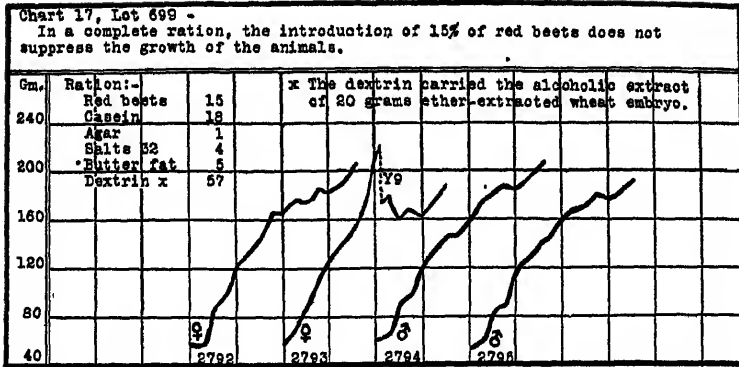
CHART 17. Proof that the failure of growth in Lot 586, Chart 16, was not due to any unfavorable factors in the diet introduced with the 15 per cent of red beets is brought out by this lot of animals. When added to a complete diet no prejudicial effects were observed It must be remembered, however, that this evidence is not absolute as in a satisfactory ration factors slightly detrimental in action would be obscured by the greater powers of resistance of the animal under these conditions. It can be safely concluded that red beets are very low in or practically free from the fat-soluble vitamine.

Fat-Soluble Vitamine in Parsnips.

The roots were sliced and then dried at room temperature in an air current. While fairly dry, they were sufficiently hydroscopic to remain tough until dried over calcium chloride; after that, they could be readily pulverized. The comminuted preparation was of a faint but clear yellow color which appeared to have greater permanency than the yellow of the rutabagas which as already noted bleached out rapidly.

Chart 18, Lot 691:-
15% of parsnips does not furnish a sufficiency of fat-soluble vitamine for continued growth.
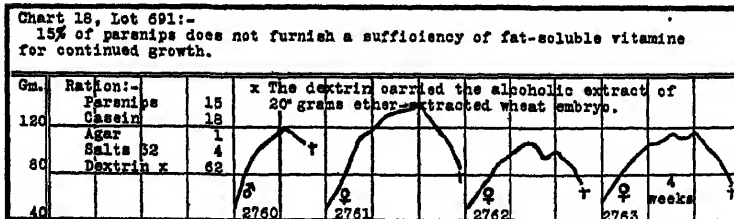
CHART 18 In two experimental lots of which the record of only one, viz 691, which showed the most growth is here reproduced no evi-

The tubers were bought on the open market and were of medium size.   They were washed free from dirt and then sliced without paring   Those fed raw were dried at room temperature while those fed cooked were autoclaved at 15 pounds pressure from 60 to 75 minutes.   Generally after cooking they were dried in an oven at 90°C.   An exception to this procedure is to be noted, Chart 24, Lot 719, where they were dried in an air current at about 30°C.

Chart 20, Lot 178.–
   15% of raw potatoes furnishes a sufficiency of the water-soluble vitamine for long continued growth.

Ration:–
Raw potatoes 15
Salts 4
Agar 1
Casein 18
Butter fat 5
Dextrin 57
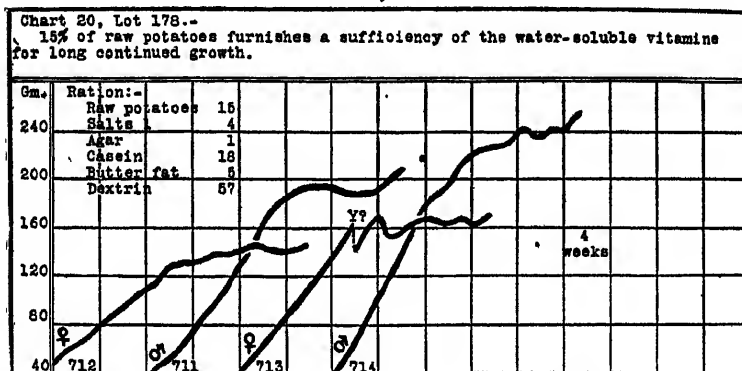
CHART 20.   Potatoes furnish us with another instance of the relatively large amounts of water-soluble vitamine present in proportion to the animal's requirements for growth.   This does not necessarily mean that the amount present in their diet is generally much larger than the animal requires for its physiological well being—though evidence points in this direction—but it does mean that on a diet of roots the animal is certain to feel the need of other constituents before that of the water-soluble vitamine

Chart 21, Lot 161:–
   Without additional fat-soluble vitamine 15% of raw potatoes will not allow growth to continue.

Ration:–
Raw potatoes 15
Salts 1
Agar 1
Casein 18
Dextrin 62
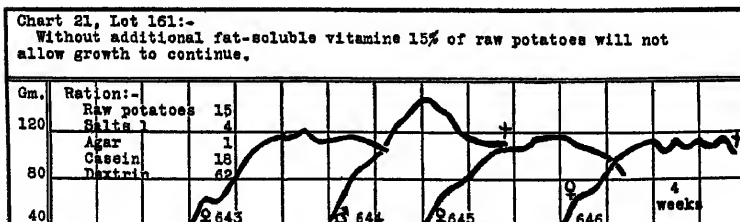
CHART 21   When the ration fed to Lot 178, Chart 20, was not provided with fat-soluble vitamine in addition to that furnished by the 15 per cent of raw potatoes, failure in nutrition results   Here again, then, we have an instance where, relative to the needs of the animal for growth, a

deficiency in the fat-soluble vitamine becomes especially evident. After failure to grow had been decisively indicated the ration was changed to the extent that five parts of dextrin were replaced by five of butter fat Before the effect of this vitamine addition had become evident the animals died, death being directly due to their poor nutritive-condition.

Chart 22, Lot 242.-
25% of raw potatoes does not furnish enough fat-soluble vitamine.
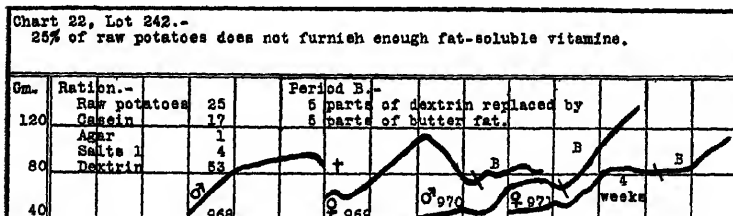
CHART 22 With such amounts of fat-soluble vitamine as are present when 25 per cent of the ration is supplied as raw potatoes, the observed growth performance was less than on 15 per cent (Chart 21, Lot 161) This is due to the fact, as was discovered later when large amounts of raw potatoes were fed, that raw potato starch is digested with great difficulty by the rat. Therefore, as the amount of potato in the ration was increased, such improvement as would result from any additional amount of the fat-soluble vitamine present was masked by the unfavorable effect of the decrease in the amount of available energy. There remains, however, absolutely no doubt that the failure of continued maintenance on this ration was primarily caused by a lack of the fat-soluble vitamine as Rats 968, 969, and 971 all contracted xerophthalmia while on the ration

In the light of what has been said the improvement in the condition of the rats when butter fat was added as indicated on the chart should not be accepted without reserve as being due to the fat-soluble vitamine thereby added. In part the beneficial results are to be attributed also to the increased intake in the required amount of energy of which the animal was otherwise deprived by the indigestibility of the starch.

Chart 23, Lot 336.-
60% of cooked potatoes may allow long continued though subnormal growth indicating the presence of the fat-soluble vitamine.
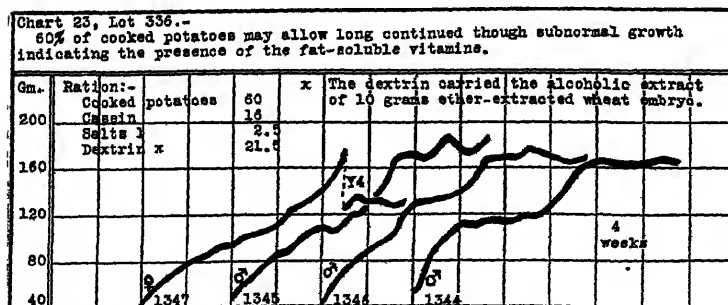
CHART 23. As when even low percentages of raw potatoes were present in the ration there was marked evidence of their indigestibility it

obviously was an impossibility to attempt to demonstrate the presence of the fat-soluble vitamine in such potatoes   Much as we felt disinclined, we were forced to cook them to render them digestible. On potatoes so treated, much to our surprise, the rats continued to grow for a considerable period of time and later maintained themselves for a period of 23 weeks with but 60 per cent of potatoes in the ration as the source of the fat-soluble vitamine   At the end of this time none of the rats gave signs of impending nutritive failure

Chart 24, Lot 719:-
   Potatoes do not always carry sufficient fat-soluble vitamine for even sub-normal growth (Chart 23).

Gm.	Ration:-											
160	Potatoes	79.5										
	Casein	16										
	Salts 32	2.0										
120	Salts 35	2.5										
80												
40				♂2872	♀2873		♀2874	♀2875				
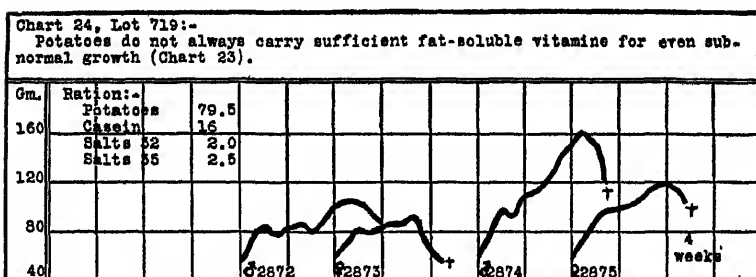
CHART 24   That much reliance is not to be placed on the general occurrence of the fat-soluble vitamine in the potato is brought out in this experiment. The potatoes used were from a different lot, but were prepared for feeding as those used for Lot 330 with the exception that later they were air-dried so that the treatment to which they were subjected was even milder than that used in the other instance and therefore more of the original vitamine content should have been preserved   The general miserable condition of these animals testified to the fact that even with 19 per cent more of potatoes in the ration as compared with Lot 336 there was far less fat-soluble vitamine demonstrable   We are forced to the conclusion that there is considerable variation in the fat-soluble vitamine content of potatoes, a fact the importance of which we did not appreciate until similar observations of variations were obtained with other plant materials   In general it is believed safe to infer that potatoes may contain enough of the fat-soluble vitamine for normal growth, but generally they can be considered poor in their content of this dietary essential.

Fat-Soluble Vitamine in Mangels.

The mangels used in these experiments were a variety known as sugar mangels which are considered of superior value on account of their higher sugar content to the ordinary mangels used as fed by the animal husbandman and so extensively used by the Germans for human food during the time of food scarcity in 1917. They were washed, pulped on a power beet rasp, and then

dried in an air current at room temperature. In air-dried condition they were readily pulverized and incorporated in the ration.

Chart 25, Lot 245.-
  25% of mangels does not carry enough water-soluble vitamine for growth.

Gm.	Ration:-											4 weeks
120	Mangels	25										
	Casein	17										
80	Butter fat	5										
	Agar	1										
	Salts I	4										
40	Dextrin	48										

980   981   982   983
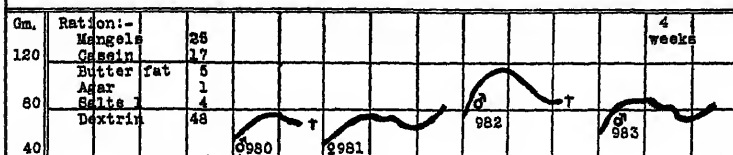
Chart 25. Unlike carrots, rutabagas, dasheens, potatoes, beets, and parsnips 25 per cent of mangels does not furnish the rat with the required amount of water-soluble vitamine. In an otherwise satisfactory ration the lack of water-soluble vitamine was indicated by the failure of the animals to grow and in case of Rat 980 by an attack of convulsions characteristic of polyneuritis which terminated fatally.

Chart 26, Lot 405:-
  The presence of mangels in a diet to the extent of 25 per cent exercises no special prejudicial action.

Gm.	Ration:-											
200	Mangels	25										
	Casein	17										
160	Salts I	4										
	Agar	1										
	Wheat embryo	3										
120	Butter fat	5										
	Dextrin	45										
80												
40												4 weeks

1620   1623   1621   1622
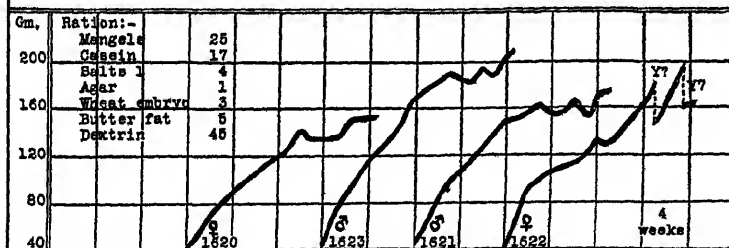
Chart 26. The growth performance of this group of rats on a ration different from that of Lot 245, Chart 25, only in that three parts of dextrin were replaced by ether-extracted wheat embryo—well known to be rich in the water-soluble vitamine—demonstrates that no mistake was made in attributing the cause of failure in Lot 245 to a deficiency of the water-soluble vitamine.

Chart 27, Lot 291 -
25% of mangels does not allow normal growth to occur in a ration otherwise complete but for the fat-soluble vitamine.

Gm.	Ration.—			x	The dextrin carried the alcoholic extract of 10 grams of ether-extracted wheat embryo.					
120	Mangels	25								
	Casein	18								
80	Salt 1	4								
	Agar	1								
40	Dextrin x	53								4 weeks
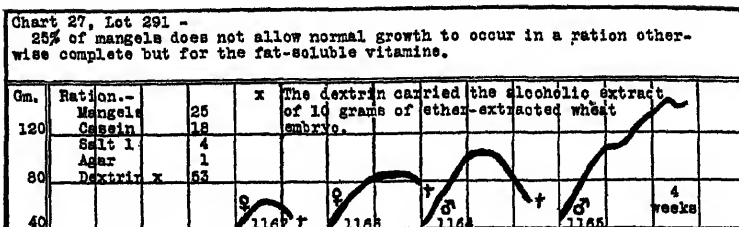
1164 ♀ ♂   1165   1164 ♀ ♂   ♂ 1165

CHART 27.   Besides their poverty in the water-soluble vitamine mangels are also deficient in the fat-soluble vitamine as indicated by the results of this experimental group   Rats 1164, 1166, and 1167 died when in a very miserable condition due to a poor nutritive state of the skin   The general condition of Rat 1165 was no better than the others, but it maintained its weight better for the time that the experiment was continued.

Fat-Soluble Vitamine in Sugar Beets.

Sugar beets prepared for feeding by pulping and then drying in an air current at room temperature and finally over calcium chloride indicated the same general vitamine relations as the mangels.

Chart 28, Lot 244:-
Sugar beets like mangels are very poor in water-soluble vitamine as indicated by failure of growth on this ration carrying 25% of the dried roots.

Gm.	Ration.—									
120	Sugar beets	25								
	Butter fat	5								
	Casein	17								
80	Agar	1								
	Salts 1	4								
40	Dextrin	48								4 weeks

976 ♀   977 ♀   ♂ 978   ♀ 979
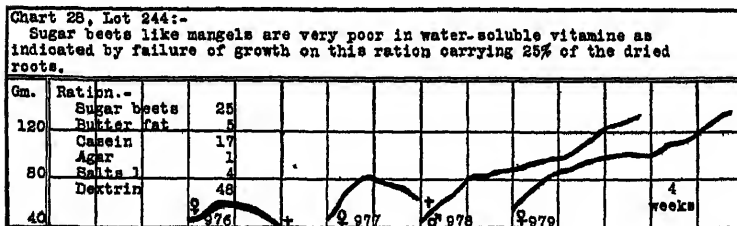
CHART 28   Rats 976 and 977 failed to live longer than 8 weeks on a ration dependent for the water-soluble vitamine on the amount introduced with 25 per cent of sugar beets   Their death was directly due to this deficiency as both died after having shown severe convulsive symptoms common in attacks of polyneuritis   Rats 978 and 979 did not succumb to this deficiency, neither was their growth satisfactory.   Such variations are merely instances of certain individuals being better able to withstand an unfavorable environment

Chart 29, Lot 292:-
When the water-soluble vitamine deficiency was corrected, growth was then prevented by lack of the fat-soluble vitamine.

Ration.-
Sugar beets 25
Casein 17
Agar 1
Salts 1 4
Dextrin x 53

x The dextrin carried the alcoholic extract of 10 grams ether-extracted wheat embryo.
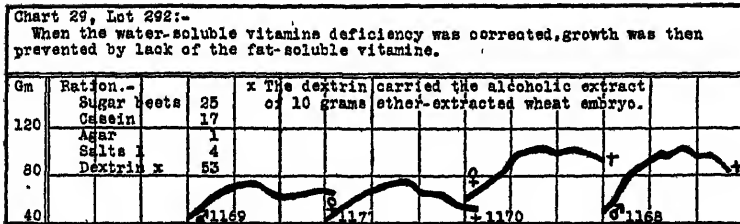
CHART 29   The evidence for a fat-soluble vitamine deficiency in a ration dependent for this dietary essential on 25 per cent of sugar beets was so pronounced that no further experiments to determine this were inaugurated; Rat 1169 was totally blinded by the xerophthalmia, Rat 1170 contracted xerophthalmia in one of its eyes before death, and Rat 1171 in both eyes.   It was only in the case of Rat 1168 that no symptoms of vitamine deficiency outside of failure to grow were observed

Fat-Soluble Vitamine in Yellow Sweet Potatoes.

Next to the Irish potato there is no tuber or root crop which enters into the make-up of the human diet to such a large extent as the yellow sweet potato, especially when it is in season as its poor keeping qualities more than anything else has prevented its continued use.   The favor with which it is received in the American home makes a comparison of its dietary properties with the Irish potato important.

The potatoes used for most of the following investigations were peeled potatoes that had been dried at a temperature of 50–60°C. In three instances air-dried potatoes were fed for purposes of comparison.

Chart 30, Lot 477:-
15% of oven-dried sweet potatoes in an otherwise approximately vitamine-free ration does not furnish enough vitamines for growth.

Ration:-
Sweet potatoes 15
Casein 18
Salts 1 4
Agar 1
Dextrin 52
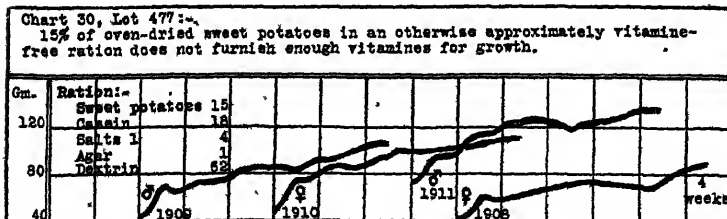
CHART 30.   15 per cent of sweet potatoes as a source of vitamines in the diet of young growing rats led to a very peculiar rate of increase in weight.   Growth at a very slow rate was continued for 22 weeks during which there were no signs of polyneuritis or xerophthalmia   The form of the curve, together with the general body condition of the animal—

recorded as good with the exception of Rat 1908 which was designated
fair—is most suggestive of a water-soluble vitamine deficiency Evi-
dently there was just enough vitamine present to maintain the animals.
It remained to establish whether or not at this level of sweet potato
feeding a deficiency of the fat-soluble vitamine did occur.
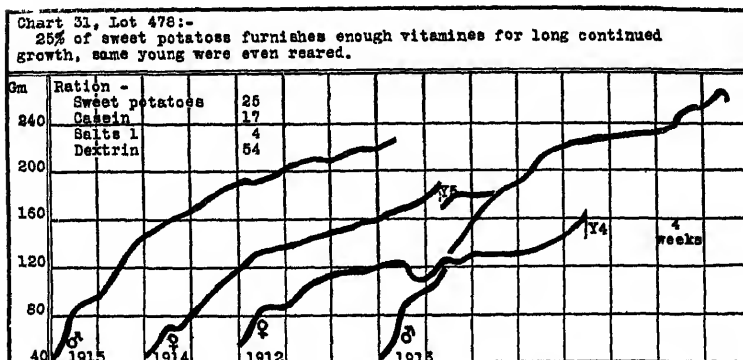
CHART 31   That growth on sweet potato vitamines becomes possible
is shown when such an amount of sweet potatoes is introduced into the
ration that it makes up 25 per cent of the total   This experimental group
indicates that in the previous lot (Lot 477, Chart 30) failure of growth
was not primarily due to any unsatisfactory factors introduced into the
ration with the potatoes and that therefore it must have been due to a
lack of vitamine. The performance of this group of animals is rather
remarkable, not only in the growth responses, but also in the fact that
one animal, Rat 1914, raised young. She raised three out of a litter of
five to an average weight of 37 gm in 5 weeks. Though undersized they
were in good condition
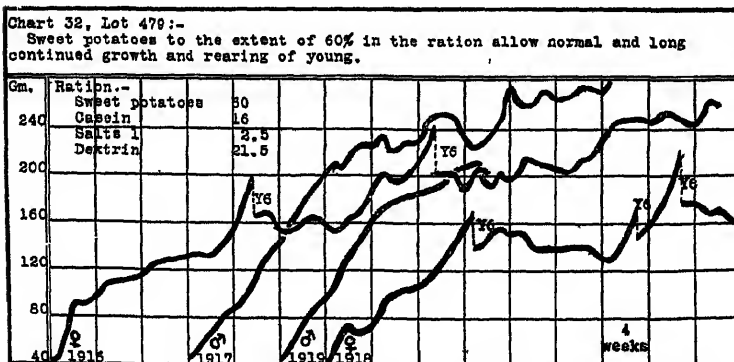
CHART 32   With the per cent of sweet potatoes increased to 60 the
rate of growth was more nearly what could be considered normal for the

rats of our colony   But as noted on the chart there occurred numerous
variations in the weights of the individuals which are indicative of digestive
disturbances as often indicated by the bloat observed from time to time.
That the vitamine requirement for growth must have been generously
satisfied must be inferred from the record of reproduction.   Rat 1916
raised five out of a litter of six young to an average weight of 48 gm. in
5 weeks and 4 days, and all of six young from a second litter to an
average weight of 42 gm in 5 weeks (see Plate 1)   The other female,
Rat 1918, raised two young out of her first litter of six to an average
weight of 38 gm. in 7 weeks and all of eight young in her third litter to an
average weight of 61 gm. in 7 weeks and 4 days.   The second litter was
not raised for reasons unknown as they disappeared from the cage a few
days after birth, evidently having been consumed by the mother.   There
certainly is nothing to indicate that the sweet potato cannot be an im-
portant source of vitamine in the diet   None of these animals showed
any signs of premature senility at 10 months which is further evidence
of the satisfactoriness of the diet

Chart 33, Lots 269 and 476:-
When the water-soluble vitamine deficiency is taken care of, the high
content of fat-soluble vitamine in sweet potatoes becomes demonstrable.

CHART 33   The experimental results of two separate feeding trials
indicate that the primary vitamine deficiency in our sweet potato material
was the water-soluble vitamine deficiency.   When this was corrected by
the addition of an alcoholic extract of ether-extracted wheat embryo
fairly good growth, together with rearing of the young, became possible.
Neither the growth of the original lots nor the growth of the offspring
was normal, but nevertheless the performance was remarkable as growth
was long continued   Rat 1077 in the 19th week developed an edematous
eye and Rat 1078 an inflamed eye   These abnormal conditions were
temporary and later entirely disappeared   They were diagnosed as not
being caused by a fat-soluble vitamine deficiency and, as the attacks
were not of long duration and were not accompanied by great losses in
weight, they were taken as being due to trauma.   This later appeared
entirely justifiable in view of the success in reproduction.   Rat 1076
raised four young to an average weight of 59 gm. in 9 weeks, and Rats

1904 and 1907 five young to an average weight of 44 gm in 7 weeks and 4 days These must not be taken as instances of satisfactory rearing of young, as their rate of development was only approximately one-half of what it should be, it was remarkable that young were raised at all

Chart 34, Lot 684:-
There is no danger of introducing demonstrable amounts of the fat-soluble vitamine with our water-soluble vitamine preparations (Chart 8).
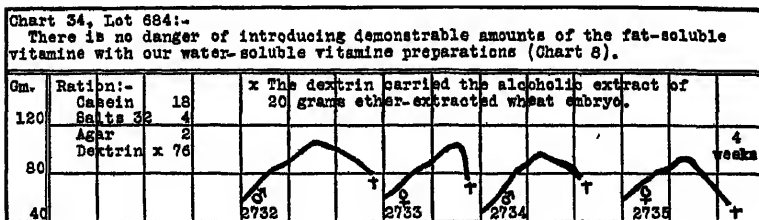
CHART 34. While we had demonstrated once before (Chart 8) that our water-soluble vitamine preparation contains little if any fat-soluble vitamine, the demonstration, on account of the importance of the data just discussed, was here repeated. On a ration containing the alcoholic extract of 20 gm of ether-extracted wheat embryo as the sole possible source of the fat-soluble vitamine all the rats succumbed within 3 months and all had infected eyes, although Rat 2732 owed its death directly to an abscess on its jaw and Rat 2733 to a pulmonary infection The results prove that the growth in Lots 269 and 476, Chart 33, could not possibly have been due to the high fat-soluble vitamine content of the water-soluble vitamine extract.

Chart 35, Lot 574:-
15% of air-dried sweet potatoes furnishes little if any more vitamines than 15% of oven-dried material (Chart 30).
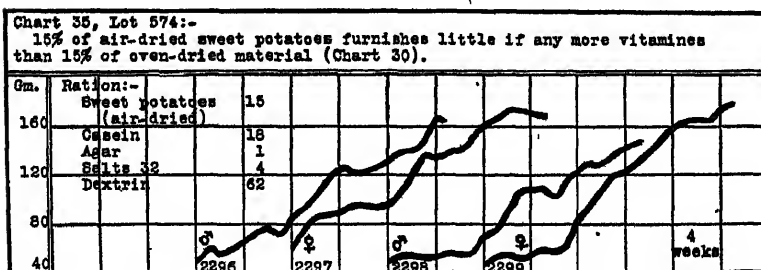
CHART 35. In spite of the data on the stability of the water-soluble vitamine now at hand in the literature, it cannot be taken for granted that the vitamine is left intact in the process of drying where temperatures higher than room temperature are employed The sweet potatoes used in this and the following two lots were obtained on the local market. They were peeled, then sliced, and dried in an air current at room temperature. On them, at a level of 15 per cent as the source of vitamines, growth was slow and indicated a lack of vitamines as in Lot 477, Chart 30, of the oven-dried material

Chart 36, Lot 690:—
In air-dried sweet potatoes as in the oven-dried material the deficiency of the water-soluble vitamine is most prominent
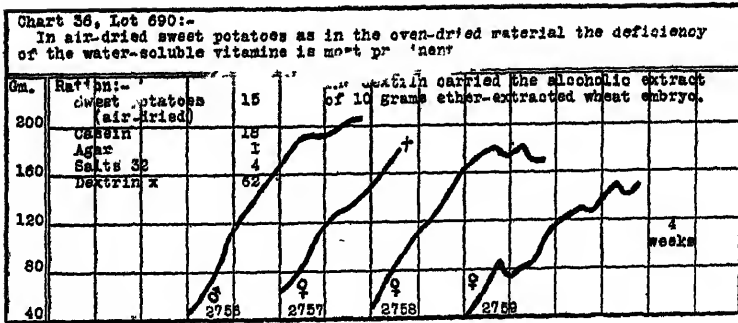
CHART. 36. 15 per cent of the air-dried sweet potatoes when fortified with water-soluble vitamine enables more rapid growth to occur than without this addition. The experiment has not yet been concluded, but the indications are that the order of magnitude of the vitamine deficiencies of a vitamine-free ration having its vitamine introduced with small percentages of air-dried sweet potatoes are of the same character as those of the oven-dried material.

CONCLUSION.

From the data of the present series of investigations it is seen that the vitamine relations in roots may be of a widely differing character. With 15 per cent of the diet made up of roots as the source of the fat-soluble vitamine we have in the case of the yellow sweet potato and carrot normal growth and even rearing of the young made possible, but in the case of the rutabaga, dasheen, red beet, parsnip, potato, mangel, and sugar beet complete failure resulted. In fact, in some instances failure at such higher levels as were dried—25 per cent in the case of the mangel and sugar beet and 83 per cent in case of the dasheen—was also observed.

Likewise, from the data here presented—which are representative of a large number of experiments—the conclusion seems warranted that tubers and roots are not necessarily to be classed with food materials grossly deficient in their fat-soluble vitamine content. While in some instances it is true that there is little or no fat-soluble vitamine demonstrable, in other instances there is enough present to warrant their classification with respect to their content of this dietary essential with leafy materials rather than with our cereal grains such as maize, wheat, barley, or oats.

It must not be taken for accepted that absolute comparisons of the amounts of the vitamine to be found in different plant materials are possible as our knowledge of the occurrence of the vitamine is too limited   At any rate it is probable that not until we know something of the function of this vitamine in the plant kingdom, or at least know something of its association with specific principles or physiological processes that general statements on the basis of such limited data as here presented will be warranted.   The danger of drawing conclusions from limited data is brought out graphically in the case of the experiments carried out on potatoes   In only one out of two instances was the presence of the fat-soluble vitamine demonstrated when different samples were fed at high levels   We believe that this is only one of numerous instances of variation in the natural occurrence of vitamine which later may be easily understood as their physiological rôle is appreciated   We believe, however, that our general conclusions in regard to the especially high fat-soluble vitamine content of carrots and yellow sweet potatoes as compared with red beets, parsnips, rutabagas, sugar beets, potatoes, mangels, and dasheens will not need qualification

The water-soluble vitamine relations as brought out in our experiments are also worthy of some comment   In the case of the carrot, rutabaga, and dasheen 15 per cent of the material furnished enough of this vitamine for growth   Of sweet potatoes a somewhat larger amount was necessary, but of the sugar beet and mangel, even as much as 25 per cent of the ration gave no evidence of furnishing this compound.

In comparing the relative amounts of fat soluble vitamine and water-soluble vitamine occurring in the various materials studied it is noteworthy that there is no evident relation between them as measured by the amount required to enable the rat to grow. While in dasheens no fat-soluble vitamine could be demonstrated no matter what the amount fed—though 15 per cent furnished enough water-soluble vitamine—in carrots on the other hand enough of both the fat- and water-soluble vitamine was furnished by an amount equivalent to 15 per cent of the ration.   Again, in mangels and sugar beets both vitamines were present in such small amounts, if at all, that they could not be demonstrated when fed at a 25 per cent level   From the standpoint of plant

physiology it is difficult to surmise just what these relations signify, but most certainly it does not appear justifiable to associate generally great physiological activity with an abundance of vitamine.

Acknowledgments and thanks are due to Dr J. S. Caldwell, Dr R. A. Young, and Dr. P. H Dorsett of the Bureau of Plant Industry, Washington, D C., for cooperation in the experiments to the extent of supplying the authors with dasheens and sweet potatoes suitable for these experiments

Fig. 1   Rat 1916 was raised on a ration which derived its entire content of vitamine, both fat- and water-soluble, from sweet potatoes which made up 60 per cent of it (Chart 32)   Started on the ration at 45 gm September 9, 1918, she weighed 215 gm June 9, 1919   As seen from the picture she was in excellent nutritive condition just after she had raised the second litter of young.

Fig 2   Two out of the litter of six young all of which were raised by Rat 1916 on the 60 per cent sweet potato ration as the source of vitamines. When photographed at the age of 5 weeks and 4 days they averaged 53 gm. in weight · Though normally on our stock ration they would have weighed this 12 days sooner, their performance on this restricted ration is none the less remarkable.
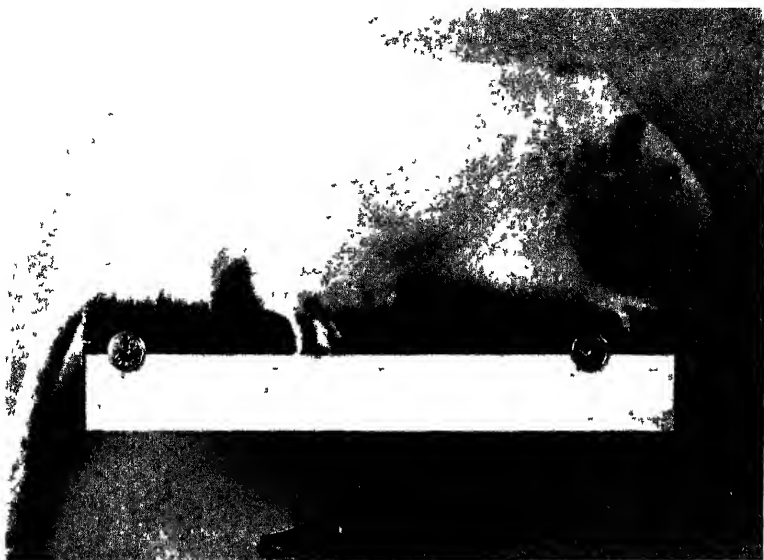
FIG 1
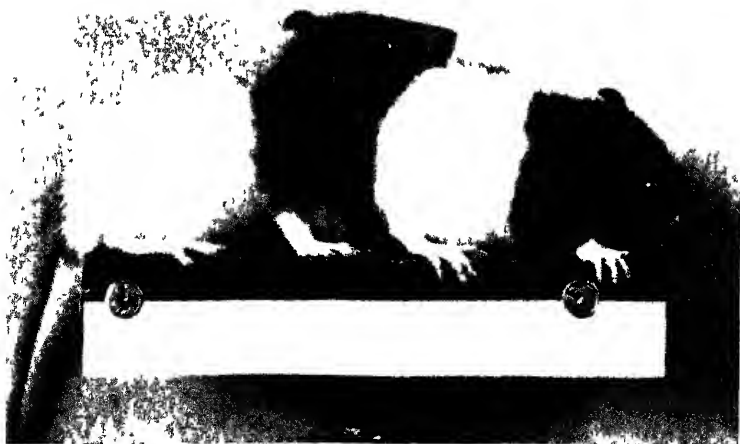
FIG 2.

(Steenbock and Gross Fat-soluble vitamine   II)

CHEMOTHERAPEUTIC STUDIES ON ORGANIC COMPOUNDS CONTAINING MERCURY AND ARSENIC.*

By GEORGE W. RAIZISS, JOHN A. KOLMER, AND JOSEPH L. GAVRON

(*From the Dermatological Research Laboratories, Philadelphia.*)

The valuable properties of mercury compounds in the treatment of spirochete infections are well known. Prior to the discovery of the dihydrochloride of diaminodioxyarsenobenzene, widely known as salvarsan, mercury compounds were practically the only remedies known for syphilis. While the modern therapy of syphilis is primarily based on the use of salvarsan, metallic mercury, and its inorganic and organic derivatives remain important aids in combating the infection. The elaboration of a mercury compound possessing more powerful spirochetocidal properties, also less toxicity for the body than any of the known mercury compounds, is of great importance. Attempts to synthesize such a compound were numerous (1). As yet none of the new organic mercury compounds has shown superior properties in the treatment of syphilis which would make it more useful than any of the old mercury preparations employed for years by physicians.

The researches of Dimroth (2), Pesci (3), Schrauth and Schoeller (4), and others suggest the advisability of dividing organic mercury compounds in three classes.

To one class belong the full complex compounds in which mercury is attached to carbon of two organic compounds as for instance in Pesci's mercury dibenzoic acid:
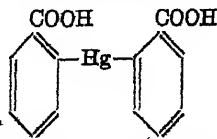
Fischer (5) and also Müller, Schoeller, and Schrauth (6) demonstrated that mercury compounds of the foregoing type are less toxic, but at the same time therapeutically less active than any other class. The reason probably lies in the fact that mercury is so firmly bound to the carbon atoms as to be almost entirely deprived of its metallic properties   This view is supported by observation that mercury cannot be split off even when these compounds are treated with hydrogen sulfide in boiling hot solution.

To the second group comprising the pseudocomplex compounds, belong mercury salts of organic acids, also compounds where hydrogen of a hydroxyl or amino group is replaced by the metal   They are characterized by the ease with which mercury is split off by the action of diluted solutions of caustic soda or hydrogen sulfide in the cold.

The last and most important group includes the so called half complex mercury compounds in which one valence of the metal is attached to the carbon atom of the benzene ring and the other to an inorganic group such as hydroxyl, halogen, or an acetic acid radical.   This group is characterized by a comparative stability.

The firmness with which mercury is bound to the organic compound is decidedly greater in half complex compounds than in pseudocomplex.   It varies with different compounds and is probably dependent upon the presence of various other groups in the benzene ring.

It is possible that there is relationship between the firmness of the position of mercury in the organic compound and the therapeutic or germicidal effect.   Such a relationship, however, can not yet be established

Two of the authors, for the past 5 years, have been engaged in a series of chemotherapeutic studies with Dr. Jay Frank Schamberg.   Organic mercury compounds were to a considerable extent the subject of these investigations   The half complex compounds were thought to be the most suitable for chemotherapeutic study and a considerable number were prepared.   Their chemical and biological properties will be the subject of this and subsequent papers   In this communication we intend to describe a class of aromatic organic arsenical compounds in which mercury was introduced.

It appeared to us particularly interesting to synthesize compounds where both mercury and arsenic were present in the molecule, as the combined effect of these elements, we thought, might be of greater therapeutic value. Only very few organic compounds containing mercury and arsenic have been described in the literature. Practically no data about their biological properties have been given.

We prepared mercurial derivatives of various phenylarsinic acids. Mercury could not be introduced into aromatic compounds containing trivalent arsenic, for instance, in arsphenamine, inasmuch as oxidation occurred almost immediately with the resulting formation of metallic mercury

EXPERIMENTAL.

3-Nitroarsanilic Acid-Mercuric Acetate, No. 76.

10 gm. of 3-nitroarsanilic acid were suspended in about 75 cc. of water and dissolved by the addition of 22 cc of 15 per cent sodium hydroxide (the amount necessary to form a disodium salt). This was mixed with a solution of 13.0 gm. of mercuric acetate dissolved in 70 cc. of water (12.1 gm = 1 mol) containing a little acetic acid to prevent the formation of any basic salt. A yellow precipitate was thrown down immediately. The whole was allowed to stand for 48 hours in the cold with vigorous mixing from time to time. Then the precipitate was filtered off, washed thoroughly with water, then methyl alcohol, and finally with ether, and dried in the desiccator over sulfuric acid.

Yield = 15.3 gm. = 77 per cent of the theoretical

	Nitrogen *per cent*	Mercury. *per cent*	Arsenic *per cent*
Analysis —Calculated	5.38	38 46	14.42
Found . .	5 73	38 38	14 52

These results are in agreement with the following formula:

$$= C_8H_9O_7N_2AsHg$$
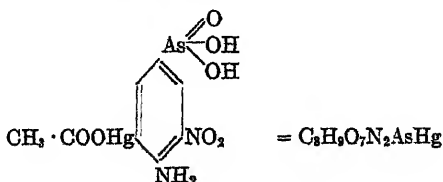
Properties —A bright yellow powder soluble in very dilute sodium hydroxide on warming   It is slightly soluble in methyl alcohol, insoluble in cold or warm ethyl alcohol, ether, and acetone   Soluble in glacial acetic acid at ordinary temperature, also in 15 per cent acetic acid on warming and in 10 per cent hydrochloric acid.

3-Nitro-4-Hydroxyphenylarsinic Acid-Mercuric Acetate, No. 95

10 gm. of 3-nitro-4-hydroxyphenylarsinic acid were dissolved using 50 cc. of water and 20 cc. of 15 per cent sodium hydroxide. To the deep yellow solution were added 12.5 gm. of mercuric acetate (12 gm = 1 mol) in 65 cc of water and a little acetic acid.   A yellow precipitate was formed which was insoluble in 0.5 per cent sodium hydroxide.   The whole was heated on a water bath for $1\frac{1}{2}$ to 2 hours when a sample dissolved completely in 0.5 per cent sodium hydroxide, thereby showing the absence of any free mercury ions.   After thorough cooling, the precipitate was filtered off, washed thoroughly with water, methyl alcohol, and ether, and then dried in a desiccator over sulfuric acid

It may be purified by dissolving in dilute sodium hydroxide and reprecipitating with acetic acid.

Yield = 13.5 gm. = 68 per cent of the theoretical.

	Nitrogen. *per cent*	Mercury *per cent*	Arsenic *per cent*
Analysis.—Calculated	. .2 69	38 39	14 40
Found . .	$\begin{cases} 2\ 61 \\ 2\ 82 \end{cases}$	38 05	14 50

$$= C_8H_8O_6N\ AsHg$$
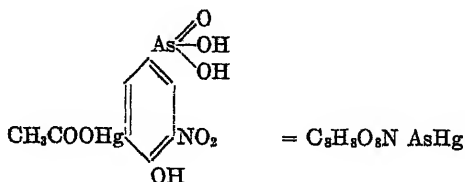
Properties —A yellow powder soluble in dilute sodium hydroxide forming a yellow solution, insoluble in both cold and warm methyl and ethyl alcohol, ether, acetone, and glacial acetic acid

3:5-Dinitro-4-Hydroxyphenylarsinic Acid-Mercuric Acetate, No. 126.

10 gm. of 3:5-dinitro-4-hydroxyphenylarsinic acid were dissolved in 10 cc. of methyl alcohol by warming on a water bath. 12 gm. of mercuric acetate (10 0 gm = 1 mol) were dissolved in 75 cc. of methyl alcohol with the addition of a few drops of acetic acid, and the two solutions mixed in the cold. The mixture was warmed on the water bath with a reflux condenser for about 10 hours until no free mercury ions could be detected by 0.5 per cent sodium hydroxide.

The precipitate was filtered off, washed thoroughly with methyl alcohol and then with ether, and dried in the desiccator.

Yield = 11 0 gm = 60 per cent of the theoretical

	Nitrogen per cent	Mercury per cent	Arsenic. per cent
Analysis.—Calculated.	4 96	35 34	13 25
Found	4 72	35 35	13 60

These results correspond with the following formula:

$$= C_8H_7O_{10}N_2AsHg$$
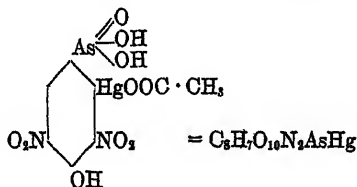
Properties —A bright yellow powder only partially soluble in dilute sodium hydroxide A pale yellow turbidity persists. It is insoluble in methyl and ethyl alcohol, ether, and acetone.

3-Amino-4-Hydroxyphenylarsinic Acid-Mercuric Acetate, No. 96.

10 gm. of 3-amino-4-hydroxyphenylarsinic acid were dissolved in 75 cc. of water and 21 cc. of 15 per cent sodium hydroxide at room temperature (because warming an alkaline solution of the above amino compound would oxidize it). A dark brown solution was thus formed. This was mixed with a solution of 14.0 gm. of mercuric acetate (13 6 gm. = 1 mol) dissolved in 70 cc. of water and a little glacial acetic acid. The mixture was kept cooled in ice water.

A light brown precipitate formed immediately.   The whole was well shaken for about ½ hour, during which time the color of the precipitate gradually darkened.   It was filtered off, washed with water, methyl alcohol, and ether, and dried in the desiccator.

It may be purified by dissolving in dilute sodium hydroxide and reprecipitating with acetic acid.

Yield = 17 5 gm. = 83 per cent of the theoretical

	Nitrogen per cent	Mercury per cent	Arsenic. per cent
Analysis.—Calculated	2 85	40 73	15 27
Found ..	2 83	40.60	. 15.25.

$$\underset{CH_3COOHg}{}\quad \underset{OH}{\overset{As{\small\nwarrow}^{O}_{OH}\ {}^{OH}}{\bigcirc}}\quad NH_2 \qquad = C_8H_{10}O_6NAsHg$$
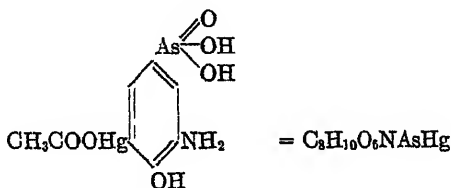
Properties.—A brown powder soluble in dilute sodium hydroxide.   This solution splits off metallic mercury within a few minutes which is deposited as a fine, gray powder   It is insoluble in the usual organic solvents, slightly soluble in cold glacial acetic acid and also 10 per cent hydrochloric acid.

3:5-Diamino-4-Hydroxyphenylarsinic Acid-Mercuric Acetate, No. 125.

10 gm. of 3:5-diamino-4-hydroxyphenylarsinic acid were dissolved in 100 cc. of water and 22 cc. of 15 per cent sodium hydroxide, thereby forming a disodium salt.   A brown solution was obtained.   This was mixed with a solution of 13 5 gm. of mercuric acetate (13.0 gm. = 1 mol) dissolved in 70 cc. of water and a little acetic acid   The mixture was kept cold by immersing in ice water and was vigorously mixed for about ½ hour, at the end of which time a sample of the precipitate dissolved completely in 0 5 per cent sodium hydroxide.   The precipitate was filtered off, washed thoroughly with water, methyl alcohol, and ether, and dried in the desiccator.

It may be purified by dissolving in 1 per cent sodium hydroxide and reprecipitating with dilute acetic acid.

Yield = 13 gm. = 65 per cent of the theoretical.

	Nitrogen per cent	Mercury. per cent	Arsenic. per cent
Analysis —Calculated.	.5 53	39.53	14.82
Found	6 06	38 87	14.84

$$\text{As}\underset{\displaystyle OH}{\overset{\displaystyle \nearrow O}{\diagdown OH}}$$

HgOOC · CH₃

H₂N   NH₂   = C₈H₁₁O₆N₂AsHg

OH
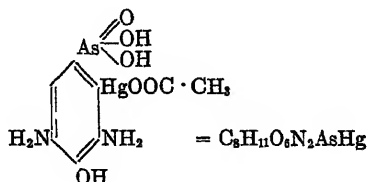
Properties.—A dark brown powder soluble in very dilute sodium hydroxide   The solution on standing splits off metallic mercury   Insoluble in the usual organic solvents   Partially soluble in cold glacial acetic acid. Soluble in 10 per cent hydrochloric acid

4-Carboxyphenylarsinic Acid- or p-Benzarsinic Acid-Mercuric Acetate, No. 127.

10 gm. of *p*-benzarsinic acid were dissolved in 75 cc. of water and 11 0 cc. of 15 per cent sodium hydroxide by warming on the water bath. This was mixed at ordinary temperature with a solution of 13.5 gm. of mercuric acetate (13.0 gm. = 1 mol) in 70 cc. of water. A white precipitate formed immediately. The whole was warmed on the water bath for about 1 hour, when a sample of the filtrate no longer showed the presence of mercury ions. The precipitate was filtered off, washed thoroughly with water, methyl alcohol, and ether, and dried in the desiccator.

Yield = 13.5 gm = 67 per cent of the theoretical.

	Mercury per cent	Arsenic. per cent
Analysis —Calculated . . .	39 68	14 88
Found	{ 39 80 { 39.75	15.13

HgOOC · CH₃

= C₉HgO₇AsHg

COOH
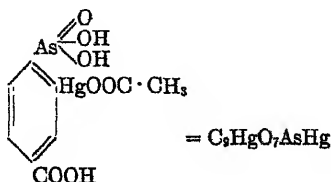
Properties —A cream-colored powder insoluble in sodium hydroxide, a yellow precipitate being formed   Soluble in dilute hydrochloric acid, concentrated sodium chloride solution, and in glacial acetic acid on warming.   Insoluble in hot or cold methyl or ethyl alcohol, ether, and acetone

Diacetyl-3.5-Diamino-4-Hydroxyphenylarsinic Acid-Mercuric Acetate, No. 97.

10 gm. of diacetyl-3.5-diamino-4-hydroxyphenylarsinic acid were dissolved in 175 cc of methyl alcohol by warming on the steam bath.   The dark brown solution obtained was mixed in the cold with 10 gm. of mercuric acetate (9 6 gm. = 1 mol) dissolved in 60 cc. of methyl alcohol   After shaking for about 20 minutes a dark gray precipitate settled out, which was filtered off and washed thoroughly with methyl alcohol and ether, and dried in a desiccator

This compound could not be further purified because of its instability.   The results obtained for mercury are not quite in accord with the assumed formula   No other formula could be suggested.   The nitrogen and arsenic values were found to agree with the assumed formula.

Yield = 15 gm = 86 per cent of the theoretical.

	Nitrogen. per cent	Mercury per cent	Arsenic per cent
Analysis —Calculated. ..	.4 75	33 90	12.71
Found	..4.65	36.40	13 05

$$= C_{12}H_{15}O_8N_2AsHg$$
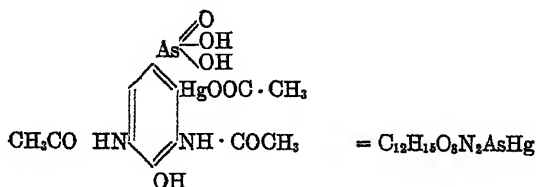
Properties.—A gray powder soluble in dilute sodium hydroxide forming a brown solution   This on standing becomes turbid, and finally metallic mercury is split off.   It is insoluble in the usual organic solvents   Partly soluble in cold glacial acetic acid

3-Bromoarsanilic Acid-Mercuric Acetate, No. 121.

10 gm. of 3-bromoarsanilic acid were dissolved in 100 cc. of
water To this were added 18 cc of sodium hydroxide and a
solution of 11.0 gm of mercuric acetate (10.5 gm. = 1 mol) in
65 cc. of water and the whole was well mixed   After heating on a
steam bath for about 3 hours, no mercury ions could be detected
by 0 5 per cent sodium hydroxide.   After cooling, the precipi-
tate was filtered, washed with water, methyl alcohol, and ether,
and then dried in the desiccator.

Yield = 15 gm = 80 per cent of the theoretical

	Nitrogen per cent	Mercury. per cent	Arsenic per cent
Analysis.—Calculated	.2 53	36 10	13 54
Found	2 74	36 15	13 52

$$CH_3 \cdot COOHg \qquad = C_8H_9O_5NBrAsHg$$
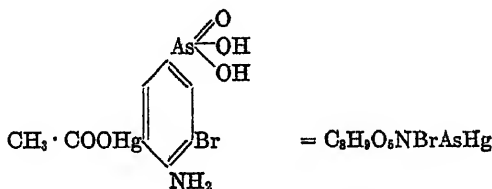
Properties.—A white powder insoluble in the usual organic solvents
Soluble in dilute sodium hydroxide, warm glacial acetic acid, and 10 per
cent hydrochloric acid.

3-Bromooxalylarsanilic Acid-Mercuric Acetate, No. 122.

10 gm. of 3-bromooxalylarsanilic acid were dissolved in 150 cc.
of water and 15 cc. of sodium hydroxide, to which was added
a solution of 9.5 gm. of mercuric acetate (8.6 gm. = 1 mol)
dissolved in 60 cc. of water. The mixture was heated on the
steam bath for about 2 hours when a sample dissolved com-
pletely in sodium hydroxide   The precipitate was filtered,
washed with water, ethyl alcohol, and ether, and dried in the
desiccator.

Yield = 8 5 gm. = 50 per cent of the theoretical.

	Nitrogen per cent	Mercury per cent	Arsenic per cent
Analysis—Calculated	.2.24	31 95	11 98
Found	2 19	{32 30 / 32 37}	11.65

$$\text{CH}_3 \cdot \text{COOHg} \overset{\displaystyle \text{As} \overset{\text{O}}{\underset{\text{OH}}{\diagup}} \text{OH}}{\underset{\underset{\underset{\text{COOH}}{|}}{\text{NH} \cdot \text{CO}}}{\bigcirc}} \text{Br}^{\cdot} \qquad = \text{C}_{10}\text{H}_9\text{O}_8\text{NBrAsHg}$$
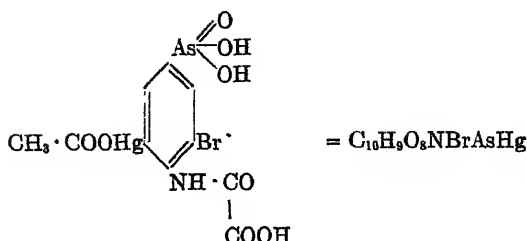
Properties —A white powder insoluble in organic solvents and acetone Soluble in very dilute sodium hydroxide. If this solution is allowed to stand metallic mercury splits off

Biological Experiments.

These new compounds were subject to a series of routine biological tests for (*a*) antiseptic and germicidal activity for staphylococci, *Bacillus typhosus*, and a spore-bearing microorganism *Bacillus anthracis*, (*b*) toxicity for white rats, and (*c*) for trypanocidal activity against infections with *Trypanosoma equiperdum* in white rats.

Results of Antiseptic or Bacteriostatic Tests.—In conducting these tests varying dilutions of solutions of the different compounds in amounts of 1 cc. were placed in sterile test-tubes and further diluted by the addition of 5 cc. of nutrient broth and seeding with 0.5 cc. of filtered, 24 hour broth cultures of the respective microorganisms. Each series of tubes was observed over a period of 5 days and the highest final dilution showing retardation of growth was recorded as the antiseptic or bacteriostatic strength of the compound. Subcultures or solid media have regularly shown that these antiseptic values do not correspond to the germicidal activity inasmuch as viable microorganisms may be recovered, but the technique is very simple, serves to bring out the finer differences in antiseptic values of the different compounds, and has proved of much value in former studies with compounds of this class for determining their influence upon bacterial activity.

The results of tests with this series of new compounds and with mercuric chloride and mercurophen are summarized in Table I and have shown the following.

1. As a general rule a higher antiseptic activity was displayed against staphylococci than *Bacillus typhosus*. This is particularly true of sodium oxy-mercury orthonitrophenolate (mercurophen) as previously described (7) and is apparent with several of the new compounds included in this series, namely, Nos. 76, 95, 97, 121, and 122. Compounds 96 and 125 did not show this peculiar and interesting superior antiseptic activity for staphylococci.

2. Two of these compounds, namely Nos. 97 and 121, have also displayed striking antiseptic activity for the bacilli and spores of anthrax, which is a distinguishing characteristic of mercurophen.

TABLE I

Results of Antiseptic or Bacteriostatic Tests of Compounds in a Menstruum of Nutrient Broth.

Substance	Mercury.	Highest antiseptic solution for		
		Staphylococcus aureus	*Bacillus typhosus*	*Bacillus anthracis*
	per cent			
Bichloride Hg..	74	1· 600,000	1: 360,000	1: 600,000
Mercurophen	53	1. 2,400,000	1: 360,000	>1: 2,400,000
No. 76.	38 3	1: 420,000	1. 180,000	1: 180,000
" 95.	38	1: 180,000	1: 42,000	1: 36,000
" 96	40 6	1: 60,000 (?)	1: 60,000	1: 54,000
" 97	36 4	1: 420,000	1. 180,000	>1: 2,000,000
" 121.	36.1	1: 480,000	1: 180,000	1: 600,000
" 122	32 3	1: 540,000	1: 180,000	1: 180,000
" 125	38.8	1: 360,000	1: 240,000	1: 120,000

Additional tests with compounds of this series which could be prepared in sufficiently concentrated solutions, have shown that a marked reduction in antiseptic activity occurs in the presence of large amounts of protein, as when the tests are conducted in a menstruum of serum. The results of tests shown in Table II were obtained by adding to varying dilutions of the compounds in water in amounts of 1 cc. an equal amount of sterile human serum and seeding with 0.05 cc. of filtered, 24 hour broth cultures of *Staphylococcus aureus;* the results indicate the highest antiseptic or bacteriostatic activities of the various compounds in a menstruum of 50 per cent human serum.

TABLE II

Results of Antiseptic or Bacteriostatic Tests of Compounds in a Menstruum of Human Serum for Staphylococcus aureus

Substance		Mercury	Highest antiseptic solution for *Staphylococcus aureus*
		per cent	
Bichloride Hg	. .	74	1: 5,400
Mercurophen	. . .	53	1·6,000
No 76	. . . .	38 3	1·6,000
" 96	. . . .	40 6	1. 4,800
" 121	. . . .	36 1	1. 6,000
" 123	. .	32 3	1: 4,800
" 125	. . . .	38 8	1: 5,400

Results of Toxicity Tests.—These tests were conducted by injecting white rats intravenously with varying doses of the new compounds per kilo of body weight and so prepared that the dose for each animal was contained in exactly 1 cc. The injections were made in a saphenous vein and at the rate of 1 cc per minute. Following injection the animals were observed for a period of 2 weeks. The results of these tests are shown in Table III and summarized in Table IV with the inclusion of mercuric chloride and mercurophen for purposes of comparison.

A few compounds were also tested by intramuscular injection into the muscles of the thigh of white rats and the results with Nos. 96 and 97 are shown in Table V.

Previous studies in these laboratories with ordinary mercurial compounds (8) have shown that toxicity is somewhat proportional to the content in mercury and this condition is apparently true of these new compounds as judged by the highest tolerated doses at the end of a 10 day period of observation. An exception to this general rule is apparent with mercurophen which is generally borne by experimental animals in doses higher than expected according to the percentage of mercury present and to which attention has been drawn in the investigations previously mentioned (7).

Results of Trypanocidal Tests.—These experiments were conducted by infecting white rats with approximately known numbers (9) of a virulent strain of *Trypanosoma equiperdum* by intraperitoneal or subcutaneous injection 24 hours before the intravenous injection of the compounds in varying dosage per kilo of

TABLE III

Results of Toxicity Tests by Intravenous Injection in Rats

Compound No	Name of compound	Mercury per cent	Weight of rat gm	Dose per kilo mg	1	2	3	4	5	6	7	8	9	10	11	12	13	14
95	3-Nitro-4-hydroxy-phenylarsinic acid-mercuric acetate	38 05	79	10	D*													
			117	8	—													
			78	4	—	—	D											
			180	2	—													
96	3-Amino-4-hydroxy-phenylarsinic acid-mercuric acetate	40 6	160	30	D													
			195	20	D													
			130	10	—	—	—	D										
			185	5	—													
97	Diacetyl-3 5-diamino-4-hydroxy-phenylarsinic acid-mercuric acetate.	36 4	107	10	—	—	—	—	D									
			98	8	—	—	—	D										
			138	6	—													
			114	2	—													
121	3-Bromoarsanilic acid-mercuric acetate.	36.15	140	30	D													
			350	20	D													
			120	10	—	—	—	D										
			185	5	—													
122	3-Bromooxalyl-arsanilic acid-mercuric acetate.	32 3	120	30	D													
			130	20	D													
			160	10	—	D												
			115	5	—													
125	3·5-Diamino-4-hydroxyphenyl-arsinic acid-mercuric acetate.	38 87	165	30	—	—	D											
			155	20	D													
			100	10	—	—	—	D										
			130	5	—	—	—	—	—	—	—	—	—	—	—	—	—	

* Died

body weight; each dose was prepared separately and contained in 1 cc. for each animal. Numerous controls were infected at the same time and in the same manner but were not injected with the compounds. Following the injections of drugs the blood of

TABLE IV

Summary of Toxicity Tests by Intravenous Injection in Rats

Compound No	Name of compound	Mercury	Highest tolerated dose per kilo	
			2 days	10 days.
		per cent	mg	mg
95	3-Nitro-4-hydroxyphenylarsinic acid-mercuric acetate.	38 05	8	2–8
96	3-Amino-4-hydroxyphenylarsinic acid-mercuric acetate.	40 6	10	5
97	Diacetyl-3:5-diamino-4-hydroxyphenylarsinic acid-mercuric acetate.	36 4	10	6
121	3-Bromoarsanilic acid-mercuric acetate	36 15	10	
122	3-Bromooxalylarsanilic acid-mercuric acetate.	32 3	5	5
125	3:5-Diamino-4-hydroxyphenylarsinic acid-mercuric acetate	38 87	10	5
0	Mercuric chloride.	74	6	3–4
0	Mercurophen.	53	10	8

TABLE V

Results of Toxicity Tests by Intramuscular Injection in Rats

Compound No	Name of compound	Weight of rat	Dose per kilo	Results in days														
				1	2	3	4	5	6	7	8	9	10	11	12	13	14
		gm	mg														
96	3-Amino-4-hydroxyphenylarsinic acid-mercuric acetate.	74	41	—	—	—	—	—	—	—	—	—	—	D			
		179	14	—	—	—	—	—	—	—	—	—	—	—			
		130	13	—	—	—	—	—	—	—	—	—	—	—			
		89	13	—	—	—	—	—	—	—	—	—	—	—			
		124	5	—	—	—	—	—	—	—	—	—	—	—			
97	Diacetyl-3 5-diamino-4-hydroxyphenylarsinic acid-mercuric acetate.	134	24	—	—	—	—	—	—	—	—	—	—	—			
		100	22	—	—	—	—	—	—	D							
		130	14	—	—	—	—	—	—	—	D						
		175	9	—	—	—	—	—	—	—	—	D					
		69	7	—	—	—	—	—	—	—	—	—	—	—			

each animal was examined daily for trypanosomes by direct microscopical examination until death occurred.

The results of these therapeutic tests are shown in Tables VI and VII. As previous studies have shown (10) mercurial compounds cannot be given in sufficient dosage to exert more than a minor and temporary influence upon experimental trypanosomiasis with this strain of trypanosomes and similar results were observed with the new compounds herein described.

TABLE VI.

Results of Therapeutic Tests with Trypanosoma equiperdum *

Compound No	Name of compound	No	Weight of rat	Dose per kilo	Results in days.						
					1	2	3	4	5	6	7
			gm	*mg*							
95	3-Nitro-4-hydroxyphenyl-arsinic acid-mercuric-acetate	1	90	12	−	+	+	+	+	+	D
		2	87	10	−	+	+	+	+	+	D
		3	84	8	D						
		4	85	6	−	+	+	+	+	+	D
96	3-Amino-4-hydroxyphenylarsinic acid-mercuric acetate.	5	145	8	−	+	+	D			
		6	220	4	−	+	+	+	D		
		7	210	2	−	+	+	+	D		
Controls.	0	8	115	0	Few.	+	+	+	+	D	
		9	144	0	"	+	+	+	+	+	D
		10	114	0	"	+	+	+	+	D	
		11	140	0	"	+	+	+	+	D	

* Rats infected by intraperitoneal injection of 180,000 trypanosomes 24 hours before the intravenous injection of the drugs.

Entirely different results may be expected in tests with rabbits infected with *Treponema pallidum* and we hope to be able to report later on this phase of the problem, when these laborious experiments are sufficiently concluded to permit of drawing comparisons and conclusions.

Further tests conducted with a new technique previously described (11), consisting in mixing in test-tubes the blood of rats showing the presence of very large numbers of these trypanosomes with an equal quantity of solutions of the new compounds

and testing for trypanocidal activity by injecting portions of the mixture intraperitoneally in rats after standing for varying periods of time at 37°C. in a water bath, have shown a high degree of trypanocidal activity on the part of several of these compounds; the results observed with two of them (Nos. 95 and 97) are shown in Table VIII.

TABLE VII

Results of Therapeutic Tests with Trypanosoma equiperdum *

Compound No	Name of compound	Weight of rat	Dose per kilo	Results in days				
				1	2	3	4	5
		gm	*mg*					
95	3-Nitro-4-hydroxyphenylarsinic acid-mercuric acetate	185	2	Few	+	+	+	D
		145	1	"	+	+	D	
		140	½	"	+	D		
97	·Diacetyl-3· 5-diamino-4-hydroxyphenylarsinic acid-mercuric acetate	180	2	"	+	+	D	
		175	1	"	+	D		
		175	½	"	+	+	D	
121	3-Bromoarsanilic acid-mercuric acetate	220	2	"	+	+	D	
		190	1	"	+	+	D	
		185	½	"	+	+	D	
122	2-Bromooxalylarsanilic acid-mercuric acetate	160	2	"	+	D		
		200	1	"	+	+	D	
		210	½	"	+	+	D	
Controls.		170	0	"	+	+	+	D
		160	0	+	+	D		
		175	0	+	+	+	D	

* Rats infected by subcutaneous injection of 800,000 trypanosomes 24 hours before the intravenous injection of the drugs

The technique is quite delicate and serves to show whether or not compounds too toxic for administration in sufficient amounts to influence experimental trypanosomiasis in living animals possess trypanocidal activity as tested by this combined *in vitro-vivo* method.

TABLE VIII

Results of Trypanocidal Tests with the Combined in Vitro-Vivo Technique.

Compound No	Name of compound	Weight of rat	Dose *	Results in days.										
				1	2	3	4	5	6	7	8	9	10	15
		gm	*mg*											
95	3-Nitro-4-hydroxy-phenylarsinic acid-mercuric acetate	84	0 001	—	—	—	—	D						
		77	0 0005	—	—	—	—	—	D					
		55	0 00025	—	—	—	—	D						
		114	0 000125	—	—	—	—	D						
		63	0 000625	—	—	—	—	—	—	—	—	—	—	
97	Diacetyl-3·5-di-amino-4-hydroxy-phenylarsinic acid-mercuric acetate	81	0 001	—	—	—	—	—	—	—	—	—	—	
		122	0 0005	—	—	—	—	—	—	—	—	—	—	
		82	0 00025	—	—	—	—	—	—	—	—	—	—	
		102	0 000125	—	—	—	—	—	—	—	—	—	—	
		92	0 0000625	—	—	—	—	+	+	+	D			
Controls	0	65	0	+	+	+	+	+	D					
		62	0	—	+	+	+	+	D					
		60	0	—	+	+	+	+	D					

* Final amount of drug acting upon the trypanosomes contained in the blood of rats.

Conclusions.

1. The new compounds herein described possess less antibacterial activity than mercuric chloride but are likewise lower in content of mercury; a number of these compounds have shown a particularly increased activity against staphylococci and a few against a spore-forming bacillus, namely *Bacillus subtilis*, as compared with mercuric chloride

2. The antibacterial activities of the new compounds tested showed a marked reduction in a menstruum rich in serum proteins.

3. The toxicity of these compounds for white rats bears a general relation to their content in mercury.

4. The new compounds possess trypanocidal activity as tested by an *in vitro-vivo* method but cannot be administered to living animals in sufficient amounts to appreciably influence the course of experimental trypanosomiasis.

Methods of Analysis.

Nitrogen was determined by the ordinary Kjeldahl method. In the cases of compounds containing nitro groups the latter were first reduced. Mercury and arsenic were determined from the same sample. 0.2 gm. was decomposed by 25 cc of concentrated sulfuric acid, diluted with water, and the mercury and arsenic were precipitated together by hydrogen sulfide. After filtering and washing, the arsenic sulfide was dissolved in ammonia and the arsenic determined gravimetrically in the usual way as magnesium pyroarsenate. The mercuric sulfide was washed successively with methyl alcohol, carbon bisulfide, and acetone, dried, and weighed. The washing with organic solvents, etc. was repeated until the weight of the mercuric sulfide remained constant.[1]

Stability.

In order to determine the degree of firmness with which the mercury was attached to the nuclear carbon, we devised the following method. 100 mg. of the compound are dissolved in a small quantity of dilute sodium hydroxide in a 25 cc. glass-stoppered cylinder and dilute acetic acid added drop by drop until the precipitate, which forms, just barely redissolves. The solution is diluted with water up to 25 cc., 5 cc of 5 per cent neutral ammonium sulfide are introduced, and the mixture is shaken. The time required for the complete precipitation of mercuric sulfide is noted If this precipitate does not come down within 30 minutes the compound is regarded as being unaffected. The mixture is then heated to 80°C. and maintained at this temperature, noting the time of the precipitation of mercury. If the result is negative after $\frac{1}{2}$ hour, a new solution of the compound is made up and treated this time with hydrogen sulfide, first at ordinary temperature, then at 80°C , and finally at its boiling temperature if necessary. None of the compounds described in this article required treatment with hydrogen sulfide. The results obtained are given in Table IX.

---

[1] This method of analysis was suggested to us by Dr W Jacobs and Dr. M Heidelberger for which we take this occasion to express our appreciation.

TABLE IX

Compound	Ammonium sulfide in cold	Ammonium sulfide at 80°C
3-Nitroarsanilic acid-mercuric acetate	Precipitated immediately.	
3-Nitro-4-hydroxyphenylarsinic acid-mercuric acetate.	Precipitated in 30 min	
3 5-Dinitro-4-hydroxyphenylarsinic acid-mercuric acetate	Precipitated immediately.	
3-Amino-4-hydroxyphenylarsinic acid-mercuric acetate	Partial precipitation	Complete after 19 min
3:5-Diamino-4-hydroxyphenylarsinic acid-mercuric acetate	Slight precipitate.	Complete precipitation in ½ hr.
Diacetyl-3, 5-diamino-4-hydroxyphenylarsinic acid-mercuric acetate.	Precipitated immediately	
p-Benzarsenic acid-mercuric acetate	" "	
3-Bromoarsanilic acid-mercuric acetate	" "	
3-Bromooxalylarsanilic acid-mercuric acetate.	" "	

SUMMARY.

1. The presence of the arsenic acid group in the molecule of organic compounds described in this paper apparently does not interfere with the entrance of the mercury group. The half complex compounds thus formed are characterized by a comparative stability in alkaline solution. The splitting off of metallic mercury occurs only in compounds containing an amino group which has also been frequently observed in non-arsenical compounds.

2. The process of formation of the organic arsenical mercury compounds and their chemical properties do not differ substantially from organic mercury compounds containing no arsenic acid group.

3. The toxic effect on the animal body as it appeared to us is mainly caused by the mercury group. The arsenic acid group neither increases nor decreases the toxicity of the compounds.

4 To our disappointment, the curative influence of these new compounds in experimental trypanosomiasis and also the germicidal effect *in vitro* were not superior to the ordinary organic mercury compounds

5. As in every other chemotherapeutic investigation the apparently negative therapeutic results with the compounds herein described cannot be accepted as definitely characteristic for the arsenical mercury compounds. Our studies were limited to a comparatively small number of compounds and further studies might result in the discovery of a powerful trypanocidal or germicidal containing both mercury and arsenic.

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